EPIGENETIC REGULATION OF DEVELOPMENTAL GENES IN EMBRYONIC STEM CELL DIFFERENTIATION

Memoria que presenta Vincenzo Calvanese
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ABBREVIATIONS

ABBREVIATIONS

AceCS1	cytoplasmic acetyl-CoA synthetase
AIM2	absent in melanoma 2
APC	adenomatous polyposis coli
Arf	alternative reading frame
AROS	active regulator of SirT1
ASCL2	achaete-scute complex homolog 2
Ash2L	ash2 (absent, small, or homeotic)-like
BER	base excision repair
BMI1	B lymphoma Mo-MLV insertion region 1
BMP1	bone morphogenetic protein 1
BRCA	breast cancer 1
CARM1	coactivator-associated arginine methyltransferase 1
CCL	human cancer cell line
CDK	cyclin-dependent kinase
CENP-A	centromere protein A
ChIP	chromatin immunoprecipitation
CBP	CREB-binding protein
CBX	chromobox protein homolog
CHD	chromodomain-helicase-dna-binding protein
CHK	checkpoint kinase
CK2	casein kinase 2
Col	colagen
CR	caloric restriction
CREB	cAMP response element-binding
DAP	death-associated protein
DBC1	deletions in tumour suppressor breast cancer 1
Des	Desmin
DLC1	deleted in liver cancer 1),
DLL4	delta-like 4
DNMT	DNA methyltransferase
DOT1	disrupter of telomere silencing
DSB	double strand break
EB	embryoid body
EED	embryonic ectoderm development
EGF	epidermal growth factor
(h) or (m)	(human) or (mouse)
ES(C)	embryonic stem (cell)
EZH2	enhancer of zeste homolog 2
FBS	fetal bovine serum
FGF	fibroblast growth factor
F-L	fibroblast-like cells
Fn1	fibronectin
FOXO	Forkhead box
FPR4	FK506-binding protein 4
GALR1	GALR1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gata4	GATA binding protein 4
GCN5	general control of amino acid synthesis protein 5
GO	gene ontology
H2ABbd	H2A Barr body-deficient
HAT	histone acetyl transferase
HCP	high CpG promoter
HDAC	histone deacetylase
HES7	hairy/enhancer of split 7
HIC1	hypermethylated in cancer 1
HIC1	hypermethylated in cancer 1
HMT	histone methyltransferase
HOXA5	homeobox A5
HP1	heterochromatin protein 1
HPH	homolog of polyhomeotic
HuR, HuD	Hu antigen R, D
ICF	immunodeficiency, centromere instability and facial anomalies
ICM	inner cell mass
ICP	inermediate CpG promoter
IKK	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase
Ink	inhibitor of cyclin-dependent kinase
iPS	Induced pluripotent stem (cell)
ISWI	imitation SWI/SNF
IVD	*in vitro*-methylated DNA
JMJ	jumonji
JNK	cJUN N-terminal kinase
KDM	lysine demethylase
KMT	lysine methyltransferase
KO	knockout
Lama1	Laminin A1
LCP	low CpG promoter
LHX1	LIM (Lin11, Isl-1 & Mec-3) homeobox 1
LINE1	long interspersed nuclear element 1
LPS	lipopolysaccharide
MART	Mono-ADP-ribosyltransferase
MBD	methyl-binding domain
MBT	malignant brain tumor
MECP2	methyl-CpG binding protein 2
MEF	mouse embryonic fibroblasts
mESC	mouse embryonic stem cell
MGMT	O-6-methylguanine-DNA methyltransferase
MHC	myosin heavy chain

MLL	mixed-lineage leukaemia
MSK	mitogen-and stress-activated protein kinase
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyl transferase
NBS1	Nijmegen breakage syndrome 1
N-CoR	nuclear receptor co-repressor
ncRNA	non-coding RNA
Nes	nestin
NES	nuclear export signal
NF-kB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHEJ	non-homologous end joining
NLS	nuclear localization signal
NODE	nanog and Oct4-associated deacetylase
NPC	neural precursor cell
NPT	normal primary tissue
NPY	neuropeptide Y
nt	nucleotide
NTT	normal tissue type
OCT4	octamer-binding transcription factor 4
PARP	poly-ADP-ribose polymerase
PAX6	paired box 6
PBL	peripheral blood lymphocytes
PcG	Polycomb group
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PHD	plant homeo domain
PGC1α	proliferator-activated receptor gamma coactivator-1 α
POU5F1	POU (Pit1, Oct1-2, Unc86) domain, class 5, transcription factor 1
PPARγ	peroxisome proliferator-activated receptor γ
PRC	Polycomb repressive complex
PRK1	protein-kinase-C-related kinase 1
PRMT	protein arginine N-methyltransferase
PUMA	p53 upregulated modulator of apoptosis
PYCARD	PYD (Pyrin) and CARD (caspase recruitment domain) domain containing
q-PCR	quantitative polymerase chain reaction
q-(RT)-PCR	quantitative (retrotranscription) polymerase chain reaction
Rb	retinoblastoma
RbBP	retinoblastoma binding protein
Rest	RE1-silencing transcription factor
RING	ring (Really Interesting New Gene) finger
RNF	ring finger protein
RSK	ribosomal protein S6 kinase
RUNX	runt-related transcription factor
SALL4	sal-like 4
SAM	S-adenosyl methionine
SCML	sex comb on midleg-like
SER-PINE1	member 1-plasminogen activator inhibitor type 1, clade E
SirT	silent mating type information regulation 2 homolog 1
SLC5A8	solute carrier family 5 (iodide transporter), member 8
SOX2	SRY (sex determining region Y)-box 2
Suv3-9h	suppressor of variegation 3-9 homolog
Suv4-20h	suppressor of variegation 4-20 homolog
Suz12	suppressor of zeste 12 homolog
SWI/SNF	mating type SWItch/sucrose non fermentable
Syp	synaptophysin
TBX3	T-box 3
Tdgf	teratocarcinoma-derived growth factor
Tcfcp2l1	transcription factor CP2-like 1
TF	transcription factor
TGFβ	tumour growth factor beta
TIMP1	tissue inhibitor of metalloproteinases inhibitor 1
TNFα	tumor necrosis factor α
TRD	transcriptional repression domain
TSG	tumour suppressor genes
UbC	ubiquitin C
UNG	uracil-DNA glycosylase
UTR	untrascribed region
UTX	ubiquitously transcribed tetratricopeptide repeat, X chromosome
VHL	von Hippel-Lindau
WB	western blot
WDR5	WD repeat domain 5
WNT6	wingless-type MMTV integration site family, member 6
WRN	gene associated with Werner`s syndrome

SUMMARY

SUMMARY

Understanding the molecular mechanisms involved in the control of cell differentiation during embryonic development is currently one of the main objectives of many laboratories worldwide. This knowledge will provide a basis for the development of new strategies in the field of regenerative medicine, one of the most promising weapons to fight many human diseases. Cell differentiation during embryonic development is controlled primarily by epigenetic factors, that is, mechanisms involved in the regulation of chromatin structure and gene expression. In this work, we studied the role of DNA methylation and certain histone modifications in embryonic stem cell differentiation.

Developmental genes are frequently silenced in human embryonic stem cells (hESC) by a bivalent histone-based chromatin mark; nonetheless, some gene promoters are hypermethylated in these cells. We thus used methylation arrays to study the role of DNA methylation in hESC differentiation. Our results confirmed that certain genes are repressed in hESC by promoter hypermethylation, and we identified some of those that are regulated by demethylation during ES differentiation. Many of these genes are developmental genes that are also hypermethylated in adult somatic stem cells and, in some cases, are hypermethylated in cancer, a common feature of tumour suppressor genes (TSG). We thus suggest that, for genes repressed by promoter hypermethylation in stem cells *in vivo*, the aberrant process in cancer could be understood as a defect in establishing an unmethylated promoter during differentiation, rather than as an anomalous process of *de novo* hypermethylation.

In studying chromatin modification in ESC differentiation, we found that the histone deacetylase SirT1 directly regulates developmental gene promoters in hESC. SirT1 proved to be precisely downregulated during hESC differentiation by a molecular pathway involving the RNA-binding protein HuR and the arginine methyltransferase CARM1. We demonstrated that SirT1 mRNA stability decreases soon after differentiation induction, as a consequence of a reduction in SirT1 mRNA-HuR binding. This reduction was associated with loss of CARM1-dependent HuR methylation at Arg217. SirT1 downregulation led to reactivation of key developmental genes such as the neuro-retinal morphogenesis effectors DLL4, TBX3 and PAX6, which are epigenetically repressed by this histone deacetylase in pluripotent hESC. We thus describe two new mechanisms for developmental gene inactivation in embryonic stem cells and for the controlled activation of their expression during lineage specification.

INTRODUCTION

INTRODUCTION

To introduce the work carried out in this PhD thesis, I will first describe the early steps of mammalian embryo development, the natural environment for cells used as the main experimental model: embryonic stem cells. After an overview of epigenetic science, I will then summarise the state of art of epigenetic mechanisms involved in stem cell pluripotency and differentiation.

Developmental biology

Multicellular organisms are composed of different specialised cells. In nearly all cases, the formation of a new individual begins with a single cell, the fertilized egg or zygote, which duplicates repeatedly to produce all the cells of the body. Development is considered the process that leads to the formation of a complete organism from a single cell, through progressive changes. As the most profound changes are observed between fertilization and birth, the study of animal development has traditionally been identified with embryology. The process of development nonetheless does not cease at birth for many organisms such as those that undergo metamorphosis, nor for mammals (including humans), whose bodies continue changing through adulthood and into old age.

Development fulfils two major objectives: it generates cellular diversity and order within each generation, and it ensures the continuity of life from one generation to the next. The formation of organized bodies has always been one of the great sources of wonder for humankind. How can matter alone construct the organized tissues of the embryo? How are all the instructions for the formation of the entire organism contained in that single cell? In the past decades, developmental biology has uncovered some secrets of this miracle.

First steps in mammalian development: pluripotency and lineage commitment. After mammalian egg fertilization, the derived zygote, the first cell of a new organism, begins to divide. These first symmetric cleavages that give rise to 2, 4, and 8 cells produce identical cells, blastomeres; these are classically considered the only totipotent cells, in the sense that each has the potential to give rise to any of the differentiated cells of the embryonic and extraembryonic tissues (Fig. I-1, left). Cells in the compact 8-cell embryo divide again to form a 16-cell morula, constituted by a small group of internal cells surrounded by a larger group of external cells (Fig. I-1, centre) (Barlow et al., 1972). The offspring of external cells give rise to the trophoblast (trophectoderm) cells, producing the tissue of the chorion, the embryonic portion of the placenta. The embryo proper is derived from the descendants of the inner cells of the 16-cell stage, supplemented by cells dividing from the trophoblast during the transition to the 32-cell stage (Fleming, 1987; Pedersen et al., 1986). These cells generate the inner cell mass (ICM), which will give rise to the embryo and its associated yolk sac, allantois, and amnion. By the 64-cell stage, the ICM (approximately 13 cells) and the trophoblast cells have become separate layers, neither contributing cells to the other group (Dyce et al., 1987; Fleming, 1987). Thus, the distinction between trophoblast and ICM blastomeres represents the first differentiation event in mammalian development. In the morula, during a process called cavitation, the trophoblast cells secrete fluid to create a cavity known as blastocoel. The ICM is then positioned on one side of the ring of trophoblast cells; the resulting structure is termed blastocyst. Isolation of the ICM at this stage and its propagation *in vitro* in appropriate conditions leads to the derivation of embryonic stem cell lines. These cells are defined as pluripotent since, although they can form any embryonic cell lineage, they have already passed the first differentiation step.

While the embryo moves through the oviduct on its way to the uterus, the blastocyst expands within the *zona pellucida (*the extracellular matrix of the egg), in which it finally creates a small opening and squeezes out through it (Fig. I-1, right) (Perona and Wassarman, 1986). Trophoblast cells can now

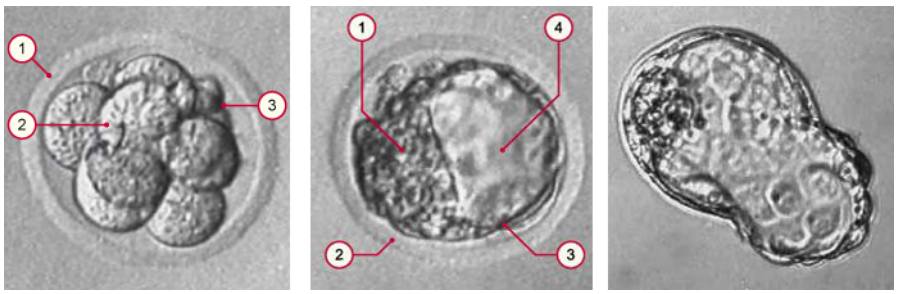


Figure I-1. First embryo stages. Morula (left) consisting of zona pellucida (1), blastomeres (2) and polar body (3). Blastocyst (centre) showing an inner cell mass (1), zona pellucida (2), trophoblast (3) and blastocoel (4). Hatching blastocyst (right). From the website www.embryology.ch

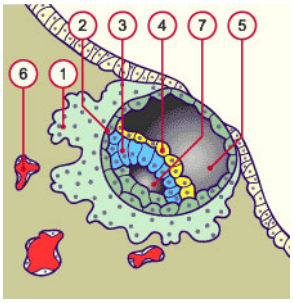


Figure I-2. Bilaminar embryo after implantation. Syncytiotrophoblast (1), cytotrophoblast (2), epiblast (3), hypoblast (4), blastocyst cavity (5), maternal blood capillary (6), amniotic cavity (7). From the website www.embryology.ch

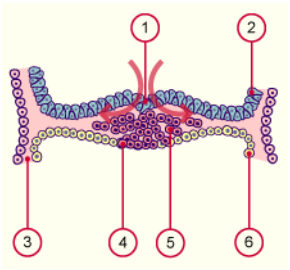


Figure I-3. Trilaminar embryo after implantation. Primitive groove (1), ectoderm (2), extra-embryonic mesoderm (3), definitive endoderm (4), invading epiblastic cells forming the intraembryonic mesoderm (5), hypoblast (6). From the website www.embryology.ch

bind to the uterine epithelium (endometrium) by expressing specific receptors and adhesion molecules; these cells produce proteases that lyse the endometrial extracellular matrix, so that the blastocyst can bury itself in the uterine wall (Brenner et al., 1989). The chorion secretes hormones that allow the uterus to retain the foetus, and further induces uterine cells to form the maternal portion of the placenta, the decidua, which becomes rich in blood vessels that will provide oxygen and nutrients to the embryo. The first segregation of cells within the ICM results in formation of hypoblast (also termed primitive endoderm) and epiblast (Fig. I-2). Hypoblast cells delaminate from the ICM to line the blastocoel, where they give rise to the extraembryonic endoderm, which forms the yolk sac. The remaining ICM tissue above the hypoblast is now referred to as the epiblast. Epiblast cells also cover all the internal surface of the cavity they are facing, the amniotic cavity. Once the amnion lining is completed, it fills with a secretion called amniotic fluid. Schematically, the amniotic cavity and primary umbilical vesicle together form two hemispheres with two layers (epi- and hypoblast) lying close to one another, representing the first embryonic structure. The epiblast alone is responsible for embryo formation; however, the hypoblast develops into a part of the extraembryonic appendages.

The bilaminar germ disk evolves to a trilaminar embryo (Fig. I-3). By day 17 in the human embryo, a thickening of the embryonic disc (primitive streak) is observed, due to cell proliferation and migration towards the median line along the rostro-caudal axis; it soon occupies roughly half the embryo. After the 19th day, the primitive streak grows through addition of cells at its caudal end. At the anterior end, a groove begins to form (primitive groove). Epiblast cells migrating through the primitive streak, between the two germinal layers, determine the formation of three new embryonic layers: the dorsal-lying cell layer facing the amniotic cavity, now called ectoderm; ventrally-lying cells that form the endoderm, which replaces the hypoblast, and cells residing between the last two, forming the mesoderm (Lawson et al., 1991). At this stage, the embryonic disk is oval-shaped and the ectoderm is bathed in amniotic fluid. Viewed dorsally, the wide end represents the rostral region and the narrower end forms the caudal region. Right and left halves are also defined.

In a few days, many different specialized cells form; the embryo itself and extaembryonic tissues are thus generated from a few identical cells. From this point onward, several decisions must be taken, not only regarding the fate of each cell, to be able to establish a defined differentiation programme, but also regarding more complex patterning mechanisms that define the structure of the entire organism. These mechanisms include definition of the three axes cited above (anteroposterior, dorsoventral, and left-right), organ formation, and integration of complex systems like the cardiovascular, musculoskeletal, and nervous systems. These processes involve multilevel organization of the molecular information, that continues to unwind as long as the embryo develops and the complexity of the system increases. This is achieved through several mechanisms, including cell-cell communication, signalling routes, and gene expression programs that are finely regulated in space and time.

As an example of a complex genetic mechanism involved in embryo patterning, we will only cite the anterior-posterior polarity specification by expression of *Hox* genes, homologues to the homeotic gene complex (*Hom-C*) of the fruit fly. Mouse and human genomes contain four copies of the *Hox* complex per haploid set, located on four different chromosomes (*Hoxa* through *Hoxd* in the mouse, *HOXA* through *HOXD* in humans) (Boncinelli et al., 1988; Scott, 1992). They are arranged in the same general order as their expression pattern along the anterior-posterior axis, with the most 3′ gene required for producing the most anterior structures, and the most 5′ gene specifying the development of the posterior abdomen. *Hox* gene expression can be seen along the dorsal axis (in the neural tube, neural crest, paraxial mesoderm, and surface ectoderm) from the anterior boundary of the hindbrain through the caudal region. Different body regions along the rostro-caudal axis are characterised by different constellations of *Hox* gene

expression, and these expression patterns create a code whereby certain *Hox* gene combinations specify a particular region (Hunt and Krumlauf, 1992). This example is particularly instructive for understanding a common leitmotif in development: only the precise modulation of gene expression patterns both spatial (in different regions of the developing embryo) and temporal (during ontologic time) enables the correct, ordered formation of an organism.

Embryonic stem cells, *in vitro* differentiation and applications. In 1998, two laboratories (Gearhart, 1998; Thomson et al., 1998) announced that they had derived human embryonic stem cells (hESC). These cells were derived either from ICM of embryos that were not implanted into *in vitro* fertilization patients, or from germ cells from spontaneously aborted foetuses. In both instances, the hESC were pluripotent, since they were able to differentiate in culture to form more restricted stem cells or differentiated cells; when injected into immunodeficient mice, they produced a teratoma containing many kinds of tissues. Since then, hESC have become a powerful model with which to understand the biology of the first developmental steps. Many efforts have been made to define the best conditions in which to maintain these cells in culture (Adewumi et al., 2007) and to induce specific differentiation. In many cases, the goal is the optimization of all the protocols for their application in regenerative medicine.

ES cells are currently generated by immunosurgery –meaning the isolation and culture of ICM cells after enzymatic removal of the *zona pellucida* and complement-mediated disruption of trophoectoderm– or whole or partial embryo culture (Moon et al., 2006). Establishment of ES cell lines depends on many factors, including culture conditions, feeder cell growth surface, culture medium composition (Ludwig et al., 2006; Nichols and Ying, 2006) and even genetic background (Yang et al., 2009).

There is hope that hESC can be used to produce new functional cells for pathological situations in which cell function is compromised. First attempts at stem cell-based therapy were tested in mouse, where ES-derived glial stem cells were transplanted into mice with a genetic deficiency in glial function, and cured the defect (Brustle et al., 1999); ES-derived neural stem cells were able to divide and differentiate into functional neurons when injected into a damaged rodent nervous system (McDonald et al., 1999). Since these demonstrations, many applications in regenerative medicine have been tested worldwide (Deb and Sarda, 2008). Research in this field has focused on overcoming the main problems for clinical application, which include differentiation towards the desired lineages (Dhara and Stice, 2008; Shiba et al., 2009; Zhang et al., 2001), efficient differentiation to eliminate the potential tumorigenicity of these cells (Blum and Benvenisty, 2008), and overcoming host rejection (Bongso et al., 2008; Drukker and Benvenisty, 2004; Grinnemo et al., 2008). The possibility of a model of in vitro-differentiated tissues of many kinds is also appealing for the world of drug discovery and validation (Jensen et al., 2009; Krtolica et al., 2009; Sartipy et al., 2007).

In the last few years, an important advance has been made through the mise-au-point of a technique to revert a fully differentiated cell back to its undifferentiated, pluripotent state. These induced pluripotent stem (iPS) cells represent a novel, attractive model system for the study of epigenetic mechanisms operating during preimplantation development. By overexpressing key embryonic transcription factors (OCT4/POU5F1 and SOX2) along with the transcriptional activators KLF4 and cMYC, the somatic epigenome is erased and its embryonic counterpart established (Hochedlinger and Plath, 2009; Takahashi et al., 2007a; Takahashi et al., 2007b). iPS cells have been derived by introducing different combinations of the above-cited and other factors, using viral or plasmid-based gene transfer methods (Stadtfeld et al., 2008b), sometimes in conjunction with small molecules (Silva et al., 2008), or by direct introduction of recombinant proteins into cells (Zhou et al., 2009). Many different cell types –differentiated and partially undifferentiated (Kim et al., 2009)– have also been used as a starting point (Aoi et al., 2008; Stadtfeld et al., 2008a), both from healthy donors and from patients (Ebert et al., 2009; Maehr et al., 2009). The relevance of iPS cells as a model system for embryonic development has been corroborated by the successful derivation of cloned mice via tetraploid complementation (Boland et al., 2009; Kang et al., 2009b). The potential application of these cells in regenerative medicine is in principle even broader than that of hESC, because any cell in the adult body can become a source of all other cells; if necessary, genetic defects could be corrected before reimplantation.

The main concern for the application of the data generated in ES and iPS cells, both in basic science and translational application, are possible alterations derived from the prolonged *in vitro* culture necessary for cell expansion to numbers sufficient for biochemical analysis. These extended culture regimens frequently lead to aneuploidy (Cortes et al., 2008), and are likely to induce aberrant mechanisms that increase

survival in culture, but are unrelated to the uterine environment. Comparison of biochemical markers between ICM cells and established mESC line demonstrated some alterations in ESC (O'Neill et al., 2006). This discrepancy might not only be the result of culture adaptation of clonal ES cells, but could also reflect heterogeneity in the ICM cell pool and might account for the low efficiencies encountered during targeted differentiation of ES cells.

Epigenetics

A first definition of epigenetics was proposed by Conrad Waddington in 1942 (Waddington, 1942) as the study of how genotypes give rise to phenotypes through programmed changes during development. New concepts were subsequently added to this original definition: an epigenetic event would be something that affects gene expression without changing the nucleotide sequence, in a way that can be inherited through cell division and possibly through gamete formation (Holliday, 1987). Today, epigenetics refers to heritable changes in gene activity and expression, as well as to stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable (http://nihroadmap.nih.gov/epigenomics/index.asp). Epigenetics in this sense would include all mechanisms for unfolding the genetic programme in processes such as development, differentiation, stress response and pathological states. Indeed, epigenetic modifications are stable but at the same time plastic, as they can be modulated by cellular or environmental factors (Reik, 2007). This nuance of plasticity is the most striking feature of epigenetics, as it enables elaboration of the genetic information and its integration with the environment. In other words, each gene is expressed in a defined space and time following the dictates of the epigenetic machinery that, by altering the physical structure of the genetic information, makes it readable or unreadable. Chromatin structure, like the genetic information itself, can be inherited by cellular progeny, creating a new and stable level of information.

Epigenetic mechanisms involve covalent chemical modification of DNA (methylation) or chromatin (histone modification) (Jenuwein and Allis, 2001) as well as other processes, mostly related to the former two, that regulate gene expression and chromatin structure (non-coding RNA, among others).

DNA, like a book, is organised into modules. All the epigenetic machinery can be seen as a complex system of enzymes or structural proteins. In response to a cell's internal and external status, these proteins are able to write the instructions in their own language for the accessibility of the basic book of each cell, i.e., the genome. Each page is represented by the nucleosome core particle. When associated with other components, higher-order nucleosomal structures are formed, like book chapters or sections. The epigenetic machinery is in charge of determining the accessibility of the pages to the readers of DNA, for example RNA polymerase. This ensures that the genetic information is stored, organized and read out in a correct spatio-temporal sequence during cell differentiation and organism development. The epigenetic code used consists of a large number of small covalent modifications on DNA or histones (Jenuwein and Allis, 2001). Among epigenetic proteins, we recognise enzymes that perform these covalent modifications, the "writers", as well as enzymes able to eliminate them, the "erasers". Finally, other proteins, the "readers" of the epigenetic code, are able to recognise these modifications and join them to the effector function, opening or closing the chapter of the book. Variations introduced into nucleosome array structures by this machinery determine differences in chromatin compaction that correlate closely with "open" versus "closed" states, which in general coincide with "active" versus "inactive" states of gene expression.

Chromatin structure

The basic repeating unit of chromatin is the nucleosome, first defined by Pierre Chambon in 1975 (Oudet et al., 1975). In 1997, the 2.8 Å crystal structure

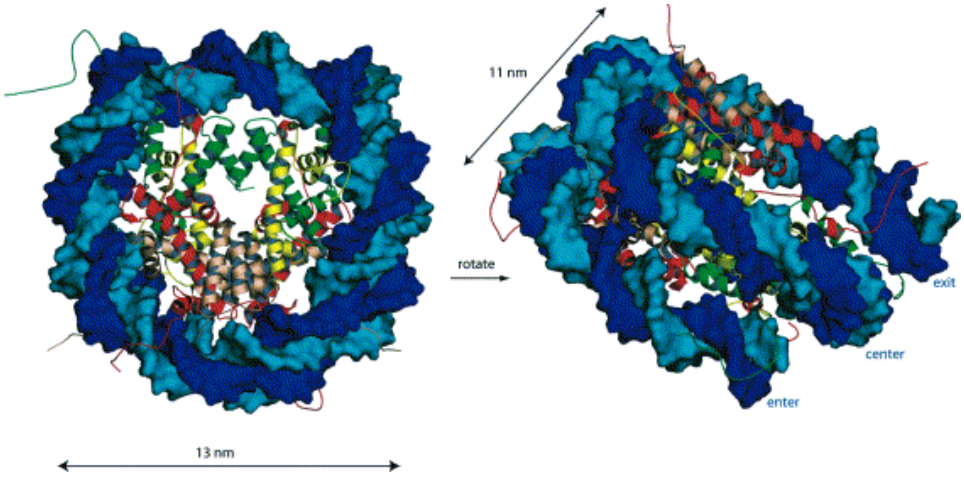


Figure I-4. Nucleosome cristal structure. DNA strands are in different colours. DNA makes 1.7 turns around the histone octamer to form a disk-like structure. Atomic structure of the nucleosome core is composed by the H3 (green)-H4 (yellow) tetramer and the H2A (red)-H2B (pink) dimer. From Khorasanizadeh, 2004.

of the nucleosome was resolved, revealing a ~147 bp double strand of DNA tightly wrapped in 1.7 left-handed superhelical turns around a core histone octamer (formed by two H2A-H2B dimers and one H3-H4 tetramer; Fig I-4).

Nucleosomes are joined by the linker DNA, together with a linker histone protein (H1) (Luger et al., 1997). Specific interactions between nucleosomes determine the folding of a nucleosomal array (the primary structure of chromatin) into a 30 nm fibre (a secondary structure) and into large-scale configurations (tertiary structures) that constitute the entire chromosome (Khorasanizadeh, 2004). Although the structure of the nucleosome core was further resolved at 1.9 Å resolution (Richmond and Davey, 2003), the precise structure of the compacted 30 nm fibre remains unknown, as its compactness impedes visualization of the spatial location of individual nucleosomes and the path of the DNA.

In general, the close interactions between DNA and histone proteins in a nucleosome lead to a high degree of structural condensation that, by default, impedes gene transcription. Histones nonetheless have positively charged, unstructured tails that protrude from the nucleosome core and can be modified in many amino acid residues. There are at least 30 possible histone modification sites for each nucleosome, eight types of modification (methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, proline isomerization and biotinylation), some of which can occur in different configurations (for example, lysine can be mono-, di- or trimethylated); a considerable number of combinations are thus possible. The information contained in the different combinations of histone marks determines the functional state of the associated DNA; this language is often referred to as the histone code (Jenuwein and Allis, 2001).

Chromatin modifications

Chromatin in eukaryotic cell nuclei is not uniformly organized, but rather contains distinguishable chromatin states: euchromatin and heterochromatin. This simplification

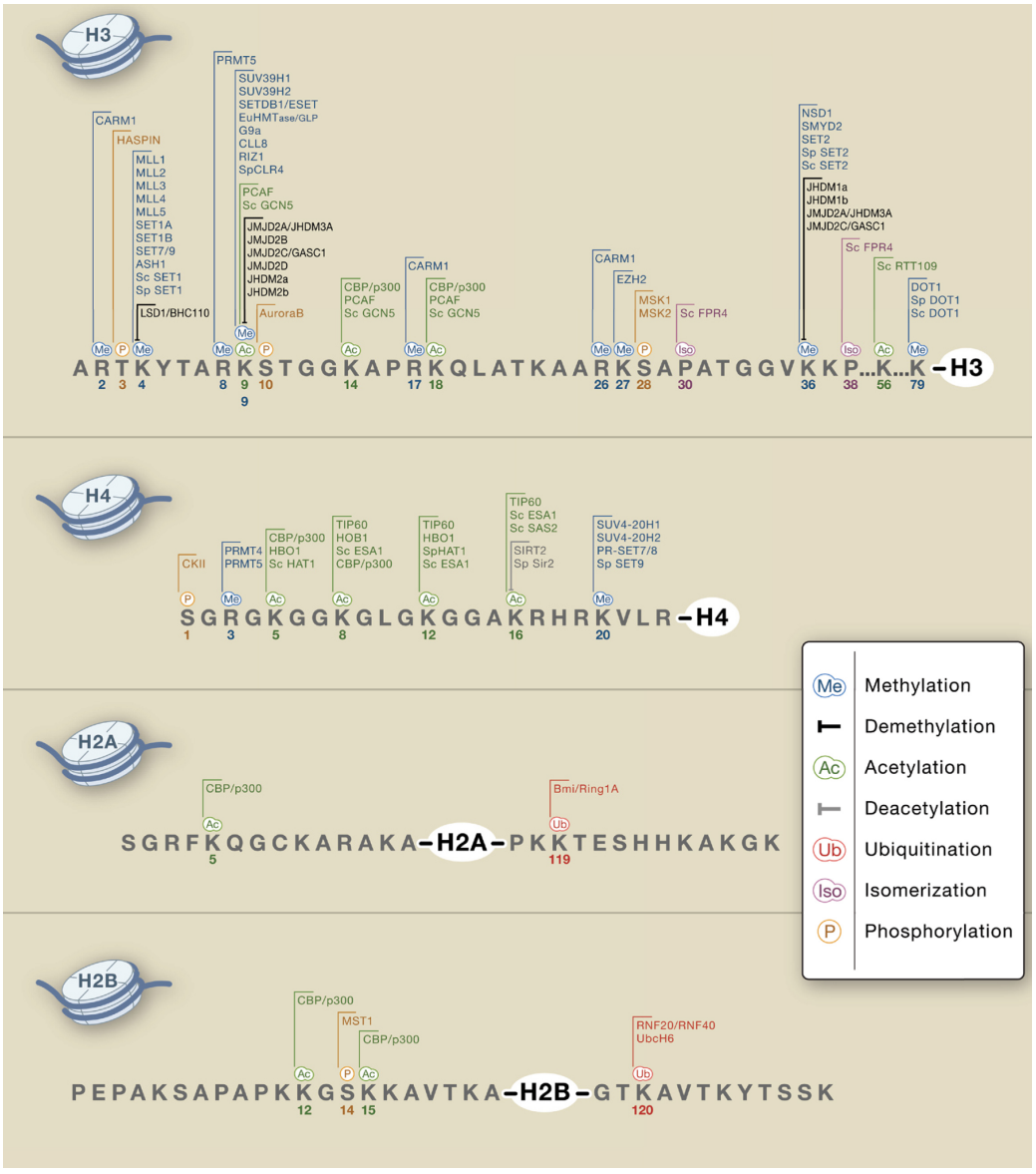


Figure I-5. A visual scheme of chromatin modification enzymes and their target residues. See text for details (from Kouzarides, 2007).

distinguishes two classes of chromatin, that in a low condensation state that is "accessible", and that with a high degree of compaction that is "inaccessible" for transcription. One of the critical functions of histone modifications is to establish and preserve these two states (Kouzarides, 2007). Although this Introduction focuses on this aspect, histone modifications also play important roles in facilitating other DNA-associated functions, such as transcription, DNA repair, DNA replication, and chromosome condensation. Many of these modifications, and the enzymes responsible for them, are shown in Fig. I-5.

They are generally classified as repressing and activating, as correlated with gene repression or induction. Some modifications can nonetheless activate or repress in a context-dependent manner. For example, methylation of H3K36 or H3K9 has a negative effect when it occurs within the promoter region, and a positive effect if in the coding region (Vakoc et al., 2005). It is thus worth keeping in mind that the final outcome of chromatin modification depends not on the single mark, but on the whole complex of modifications and their context.

Acetylation. Histone acetylation consists of the addition of an acetyl group to an ε-amino group of a lysine side chain, forming an amide bond. Acetylation of lysine 14 or 9 in histone H3 (H3K14, H3K9), and/or of H4K16 are generally associated with active gene transcription. Evidence supporting a positive link between acetylation of histone tails and transcriptional activity has long been established (Allfrey et al., 1964). Gene activation by histone acetylation has a biophysical explanation. The lysine side chain is positively charged and can bind tightly to the negatively charged DNA to form a closed chromatin structure that impedes access of transcription factors. Acetylation of lysine residues removes their positive charge and attenuates the charge interaction between histone tails and DNA. Indeed, it was demonstrated *in vitro* that acetylation of H4 at H4K16 has a negative effect on the formation of the 30 nm fibre and the generation of higher-order structures (Shogren-Knaak et al., 2006). In addition to this simple mechanism, acetylated lysines also act as docking sites for other proteins that play the role of "readers", mainly other chromatin-modifying enzymes and basal transcription machinery. One protein domain, termed the bromodomain, binds specifically to acetylated lysines (Mujtaba et al., 2007) facilitating transcriptional activation; this domain is often found in enzymes that help activate transcription, including SWI/SNF, an ATP-dependent chromatin-remodelling complex. Histones are acetylated in lysines by "writer" enzymes termed histone acetyl transferases (HAT), which transfer an acetyl group from the high-energy donor acetyl-coenzymeA to a lysine ε-amino group.

Acetyltransferases are divided into three main families, GNAT, MYST, and CBP/p300 (Sterner and Berger, 2000). Many HAT are components of large multi-subunit complexes associated with transcription initiation, recruited to promoters by interaction with DNA-bound activator proteins (Utley et al., 1998). In general, these enzymes modify more than one lysine and only a few HAT show some substrate selectivity. Histone acetylation is reversed by the "eraser" enzymatic action of the histone deacetylases (HDAC). HDAC are grouped into classes I, II and III based on sequence homology to their yeast orthologues Rpd3, HdaI and Sir2, respectively (De Ruijter et al., 2003; Gregoretti et al., 2004), and class IV, which has only one member (HDAC11). Classes I, II, and IV are referred to as "classical" HDAC and comprise 11 family members, whereas class III members are called sirtuins (Michan and Sinclair, 2007). Classical HDAC and sirtuins differ in their catalytic mechanisms. Classical HDAC are Zn^{2+}-dependent enzymes, harbouring a catalytic pocket with a Zn^{2+} ion at its base that can be inhibited by Zn^{2+}-chelating compounds such as hydroxamic acids. In contrast, sirtuins have a mechanism of action that requires NAD^{+} as an essential cofactor (Michan and Sinclair, 2007). The biology of sirtuins, and in particular of SirT1, will be discussed later in this Introduction. Similar to transcriptional coactivators with HAT activity, many transcriptional corepressor complexes such as mSin3a, NCoR/SMRT and NURD/Mi-2 contain subunits with HDAC activity (Denslow and Wade, 2007).

Phosphorylation. Phosphorylation consists of adding a phosphate to an hydroxyl (OH-) group of serine or threonine residues, thereby adding a negative charge that leads to general decondensation of the chromatin fibre (Roth and Allis, 1992). The biophysical role of histone phosphorylation has not been demonstrated rigorously *in vitro* as for acetylated H4K16 but its role in mitosis, apoptosis and gametogenesis are suggestive of this mechanism (Ahn et al., 2005; Krishnamoorthy et al., 2006). Phosphorylated residues on histones are also bound by 14-3-3 protein, linking this modification to transcriptional activation (Winter et al., 2008a; Winter et al., 2008b). As many cell signalling pathways related to protein phosphorylation, this modification is an excellent link between activation of kinase signalling pathways in response to stimuli and gene expression. For example, growth factor stimulation induces rapid phosphorylation of histone H3 at Ser 10 (H3S10) at c-Jun

and c-Fos promoters, mediated by mitogen-and stress-activated protein kinase (MSK)1, MSK2 and RSK2 kinases (Nowak and Corces, 2004; Sassone-Corsi et al., 1999; Soloaga et al., 2003). H3S10 phosphorylation is detected after activation of NFκB-regulated genes; in response to inflammatory cytokines, IκB kinase-α (IKK-α) phosphorylates H3S10 at NFκB-responsive promoters (Anest et al., 2003; Yamamoto et al., 2003). Other histone residues can also be phosphorylated: threonine 11 of histone H3 (H3T11) is phosphorylated by protein-kinase-C-related kinase 1 (PRK1), leading to transcriptional regulation in response to stimulation with androgen receptor agonists (Metzger et al., 2008).

Methylation. Methylation occurs on the functional group of lysines or arginines. This modification is functionally more complex than the others for several reasons: i) within any histone, multiple lysines or arginines can be modified, ii) individual lysine residues can be mono-, di-, or trimethylated, iii) arginine residues can be mono- or dimethylated, and these can be dimethylated in a symmetric or asymmetric fashion, iv) all the core histones can be methylated depending on physiological setting (Shi and Whetstine, 2007) and finally, v) methylation of distinct residues has opposite functional consequences on gene activation. In the case of methylation, global charge of the residue is unaffected by the modification; the final effect is thus mainly determined by different "reader" proteins that link these marks with other effectors. Histone methylation is recognized by chromo-like domains of the Royal family (chromo, tudor, MBT) and the unrelated PHD domains. Proteins with a chromodomain, such as HP1, can bind specifically to methylated lysine. HP1 is a transcription-silencing protein that interacts with HDAC; its binding to methylated H3K9 results in histone deacetylation, eventually leading to gene silencing. H3K4 trimethylation is recognized by the chromodomain protein CHD1, which can further recruit HAT to activate target gene transcription.

These various methylation reactions are mediated by "writers" termed histone methyltransferases (HMT), enzymes that use an S-adenosyl methionine (SAM) high-energy methyl-donor to transfer the methyl group onto the histone residue. Each of these enzymes has its own activation pathways and its own specificity.

Arginine methylation is catalyzed by the protein arginine N-methyltransferases (PRMT) family of enzymes (Bedford and Richard, 2005). Arginine can be monomethylated, symmetrically dimethylated and asymmetrically dimethylated, with each combination showing potentially different functional consequences (Bedford, 2007). Ten mammalian PRMT (PRMT1-10) have been identified to date. CARM1, sometimes referred to as PRMT4, can methylate histone H3 at arginine 2, 17, and 26 (H3R2, H3R17, H3R26) and enhances transcriptional activation driven by nuclear receptors, acting as a coactivator (Chen et al., 1999; Schurter et al., 2001). PRMT1 is also required for transcriptional activation by nuclear receptors, methylating arginine 3 of histone H4 (H4R3) (Chen et al., 1999; Strahl et al., 2001; Wang et al., 2001). Several coactivators capable of histone-modifying activities can cooperate synergistically; e.g., CARM1 activity can cooperate with CBP, PCAF, and p300, which are involved in histone acetylation (Daujat et al., 2002). This protein family also targets many non-histone proteins (Wolf, 2009).

Lysine methylation is operated by "writers" called lysine methyltransferases (KMT), typically specific in their histone targets; their output can be activation or repression of transcription (Kouzarides, 2007; Shilatifard, 2006). Almost all histone KMT characterized to date contain a SET domain (named after *Drosophila melanogaster* Su (var)3-9, Enhancer of zeste (E (z)), and trithorax (trx)). SET domain-containing enzymes can catalyze methylation of specific lysines on histones H3 and H4. DOT1L, the mammalian ortholog of the yeast histone methyltransferase Dot1, differs from all other known histone KMT, as it both lacks a SET domain and cannot modify free histones, instead requiring a nucleosomal substrate (Feng et al., 2002; Min et al., 2003; Ng et al., 2002; Van Leeuwen et al., 2002). DOT1L catalyzes mono-, di-and trimethylation of histone H3K79, a residue located in the nucleosome core. Although different histone KMT can share substrate specificity, it is likely that each enzyme can regulate different genes or cell processes.

Three methylation sites on histones are implicated in activation of transcription, H3K4, H3K36 and H3K79 (Berger, 2007; Kouzarides, 2007; Martin and Zhang, 2005; Shilatifard, 2006). H3K4, both di- and trimethylated, is enriched at actively transcribed genes (Bernstein et al., 2005; Santos-Rosa et al., 2002). Whereas dimethyl-H3K4 modification appears to be distributed throughout the body of active genes,, trimethyl-H3K4 modification is localized specifically at the 5′ end of these genes (Barski et al., 2007; Guenther et al., 2007; Pokholok et al., 2005). SET domain-containing protein MLL is the mammalian H3K4-specific KMT (Milne et al., 2002; Nakamura et al., 2002) and is only detected in a multi-subunit complex

with the conserved structural components RbBP5, ASH2L, and WDR5. MLL1 localizes with RNA polymerase II to the 5′ end of 90% of actively transcribed genes, where trimethyl-H3K4 is also found (Guenther et al., 2005). H3K36 methylation seems to facilitate transcription elongation. Dimethyl-H3K36 and trimethyl-H3K36 are in fact enriched at the 3' ends of transcribed genes (Bannister et al., 2005; Barski et al., 2007; Li et al., 2002b; Rao et al., 2005). H3K79 methylation correlates with transcriptional activation (Kouskouti and Talianidis, 2005; Morillon et al., 2005), and DOT1L in mammals preferentially occupies the proximal transcribed region of active genes and correlates with the presence of dimethyl-H3K79 and trimethyl-H3K79 (Steger et al., 2008).

Three methylation sites on histones are linked to transcriptional repression: H3K9, H3K27 and H4K20 (Berger, 2007; Kouzarides, 2007; Martin and Zhang, 2005; Shilatifard, 2006). Methylation at H3K9 is implicated in the silencing of euchromatic genes and in the formation of silent heterochromatin domains. Repression involves recruitment of H3K9-specific KMT (SUV3-9h1 and SUV3-9h2) and HP1 to the promoter of repressed genes. H3K27 methylation is implicated in several silencing phenomena including *HOX* gene silencing, inactivation of the X chromosome in females, and genomic imprinting (Cao and Zhang, 2004). At the core of this silencing system is the Polycomb group (PcG) proteins (Ringrose and Paro, 2004), usually found in multiprotein complexes termed Polycomb repressive complexes (PRC). PRC2 contains EZH2, EED, SUZ12, and RbBP4, whereas the PRC1 complex consists of >10 subunits, including BMI1 and the Polycomb proteins (CBX2, CBX4, CBX7, CBX8), HPH1-3, RING1-2, and SCML) (Kerppola, 2009; Levine et al., 2004). Many PcG target genes are involved in developmental patterning, morphogenesis, and organogenesis (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006b). Functionally, EZH2 is the catalytically active component of PRC2, acting as a H3K27-specific KMT (Cao and Zhang, 2004). PRC can inhibit transcription by preventing ATP-dependent nucleosome remodelling by the SWI/SNF complex as well as by directly blocking the transcription initiation machinery (Dellino et al., 2004; Francis and Kingston, 2001). PRC1 has ubiquitin E3 ligase activity that targets H2AK119, associated with gene repression (Cao et al., 2005; Wang et al., 2004). *In vitro*, PRC2 also shows KMT activity towards H1K26 (Kirmizis et al., 2004), tethering HP1 to chromatin (Daujat et al., 2005) and influencing high-order chromatin structure. EZH2 also recruits DNA methyltransferases (DNMT) to selected target genes (Viré et al., 2006). H4K20 is monomethylated by PR-Set7/Set8 (Nishioka et al., 2002; Xiao et al., 2005), whereas the murine Suv4-20h1 and Suv4-20h2 isoforms have been implicated in trimethyl-H4K20 (Schotta et al., 2004).

Several families of lysine demethylases (KDM) have been identified, which eliminate methyl marks associated with gene repression or activation; KDM usually form part of large multiprotein complexes that synergise with HDAC, KMT and nuclear receptors to control developmental and transcriptional programs. Two types of demethylase domain have been reported thus far, with distinct catalytic reactions: the LSD1 domain and the Jumonji C (JmjC) domain (Cloos et al., 2008; Klose et al., 2007). LSD1 can function both as a KDM specific for mono- and dimethyl-H3K4 (Shi et al., 2004) and mono- and dimethyl-H3K9 (Metzger et al., 2005), and for nonhistone substrates such as p53 (Huang et al., 2007). Enzyme specificity is determined partly by its association with different complexes, thereby allowing it to participate in transcriptional regulation, heterochromatin spreading and stress-induced responses (Cloos et al., 2008). At difference from LSD1, the JmjC domain-driven demethylase reaction allows demethylation of a trimethylated histone tail. There are 27 JmjC domain proteins within the human genome, 15 of which demethylate specific lysines or arginines in the H3 tail (Takeuchi et al., 2006).

Ubiquitylation. This very large modification has been found on H2A (K119) and H2B (K120 in human and K123 in yeast). Ubiquitylation of H2AK119 is mediated by the Bmi/Ring1A protein found in human PRC1 and is associated with transcriptional repression (Wang et al., 2004). In contrast, H2BK120 ubiquitylation is mediated by human RNF20/RNF40 and UbcH6 and activates transcription (Zhu et al., 2005). The mechanism of ubiquitin function is still unclear; it is likely to recruit additional factors to chromatin, but might also act to maintain chromatin open by a ''wedging'' process, given its large size.

Sumoylation. Sumoylation is another very large modification, and shows some similarity to ubiquitylation. This modification takes place on all four core histones, and specific sites have been identified on H4, H2A, and H2B (Nathan et al., 2006). Sumoylation antagonises both acetylation and ubiquitylation, which occur on the same lysine residue; consequently, this modification is repressive for transcription in yeast.

ADP ribosylation. This histone modification can be

divided into two major groups: mono-ADP ribosylation and poly-ADP ribosylation, mediated by MART (Mono-ADP-ribosyltransferases) or PARP (poly-ADP-ribose polymerases), respectively (Hassa et al., 2006). Although the function of these enzymes has often been linked to transcription, evidence that the catalytic activity is involved is lacking.

Proline isomerization. Prolines exist in either a cis or a trans conformation. These conformational changes can severely distort the polypeptide backbone. An enzyme, FPR4, isomerizes different prolines in the H3 tail (Nelson et al., 2006). Isomerization of H3P38 regulates H3K36 methylation levels, as the appropriate proline isomer is likely to be necessary for the recognition and methylation of H3K36 by the SET2 methyltranferase as well as for its demethylation (Chen et al., 2006b). The catalytic cleft of the JMJD2 demethylase is very deep and may necessitate a bend in the polypeptide (mediated by proline isomerization) to accommodate the methyl group at H3K36.

Histone crosstalk

The existence of several modifications within a short stretch of the same histone tail makes crosstalk likely. Different mechanisms have been reported, including the following: i) histone modifications cannot coexist on the same residue, and are mutually exclusive, as in the case of acetyl- and methyl-H3K9 (Fischle et al., 2003); ii) protein binding could be disrupted by an adjacent modification; i.e., phosphorylation of serine 10 inhibits HP1 binding to methylated H3K9 (Fischle et al., 2005); iii) catalytic activity of an enzyme can be compromised by modification of its substrate recognition site; isomerization of H3P38 affects the H3K36 methylation by SET2 (Nelson et al., 2006); iv) an enzyme could recognize its substrate more effectively in the context of a second modification, as is the case of the GCN5 acetyltransferase, whose action on H3 is enhanced by phospho-H3S10 (Clements et al., 2003); v) modifications on different histones can affect each other; ubiquitylation of H2B is necessary for methylation of trimethyl-H3K4 and dimethyl-H3K79 (Briggs et al., 2001; Dover et al., 2002; Sun and Allis, 2002).

Histone variants

The vast majority of histones in eukaryotic cells are expressed during the S phase and deposited on nucleosomes during DNA replication. Variants of histone H3 and H2A are nonetheless synthesised throughout the cell cycle and deposited in a replication-independent manner, conferring specialised function on nucleosomes (Henikoff et al., 2004a). Centromere protein A (CENP-A) is an H3 variant with an essential role in the assembly of centromeric nucleosomes (Henikoff et al., 2004b). It has a very different N-terminal tail from that of histone H3, presenting the opportunity for alternative posttranslational modifications. H3.3 is deposited primarily in transcriptionally active chromatin and gene regulatory sites (Chow et al., 2005; Mito et al., 2005; Mito et al., 2007). The H2A variant H2AZ is implicated in both gene activation and repression. It is localised in small regions flanking transcription start sites and larger regions proximal to telomeres or centric heterochromatin (Albert et al., 2007; Barski et al., 2007; Guillemette et al., 2005). In ESC, it binds to promoters of developmentally important genes, like the PcG protein Suz12 (Creyghton et al., 2008). H2AX has a unique C-terminal extension, important for its phosphorylation, which has an important role in DNA repair (van Attikum and Gasser, 2005). MacroH2A also has an extra C-terminal motif that binds sirtuin metabolite O-acetyl-ADP-ribose (Kustatscher et al., 2005) and is substituted in nucleosomes of the inactive X chromosome in female mammals (Costanzi and Pehrson, 1998), while also regulating key developmental genes in human male pluripotent cell autosomes (Buschbeck et al., 2009). Finally, deposition of the H2ABbd (Barr body-deficient) variant correlates with transcriptionally active chomatin (Chadwick and Willard, 2001; Chadwick and Willard, 2003).

ATP-dependent chromatin remodelling

Another group of chromatin regulators, referred to as ATP-dependent chromatin remodelling complexes, uses ATP hydrolysis to alter histone-DNA contacts (Hogan and Varga-Weisz, 2007; Saha et al., 2006). They share a common ATPase subunit of the SNF2 superfamily of DNA helicase/ATPase. ATP-dependent nucleosome remodelling factors are classed into subfamilies, depending on the presence of other domains within the ATPase-containing subunit. The four main subfamilies characterized to date are the SWI/SNF, ISWI, CHD, and INO80 subfamilies (Hogan and Varga-Weisz, 2007). These complexes mediate different nucleosome remodelling activities, ranging from subtle shifting of nucleosome positions to histone exchange or complete ejection of nucleosomes (Langst and Becker, 2004). All can basically unwrap DNA segments from the nucleosome surface and translocate them through the nucleosomes (Kassabov et al., 2002; Strohner et al., 2005). These complexes are involved in many fundamental processes

such as transcription, DNA repair, DNA replication and chromosome structure maintenance, and are finely regulated at many levels (Hogan and Varga-Weisz, 2007; Saha et al., 2006). They are often targeted by specific histone modifications and transcriptional regulators, as is the case of CHD1, which binds methylated H3K4 through its tandem chromodomain (Sims 3rd et al., 2005)

DNA methylation

DNA methylation is the simplest and perhaps best-studied epigenetic modification. In mammals, it generally consists of the addition of a methyl group to the 5-carbon of a cytosine followed by a guanine (CpG) in the DNA sequence. Throughout the mammalian genome, the CpG dinucleotide is found at a frequency much lower than expected, probably because methylation of cytosine leads to its conversion to thymine upon deamination; this impedes its recognition by the uracile-DNA glycosylase and the base-excision repair pathway. It nevertheless reaches a frequency close to that predicted in sequences that span the 5`end of many genes, regions termed CpG islands. A CpG island is thus defined by the occurrence of this dinucleotide (see methods). CpG islands are usually found in the regulatory regions of vertebrate housekeeping genes; they are often protected from methylation, enabling constitutive expression of these genes. During development, a subset of CpG islands is subjected to dynamic methylation modifications linked to tissue differentiation and formation. Once differentiation is complete, tissue-specific methylation is established in each cell type and is generally maintained throughout the cell's life. Promoter methylation accounts for only a small part of overall genome methylation; roughly 70% of all CpG are methylated and the majority are located in repetitive sequences such as endogenous retroviruses, L1 elements and Alu elements, most of which are derived from transposable elements (Yoder et al., 1997). Methylation maintains these sequences silent, making the event of amplification and new insertion in the genome extremely rare (Callinan and Feinberg, 2006). It has in fact been proposed that DNA methylation might have evolved as a genome-defence system to prevent the chromosomal instability, translocations and gene disruption caused by reactivation of these transposable DNA sequences (Yoder et al., 1997).

At least two additional genetic mechanisms, genomic imprinting and X chromosome inactivation, rely on DNA methylation in normal cells. Genomic imprinting occurs in some genes whose expression is always restricted to either the maternal or the paternal allele; imprinting requires DNA methylation at one of the two parental alleles to ensure monoallelic expression. A similar gene-dosage reduction is involved in X-inactivation in females (Heard and Disteche, 2006).

The "writers" of these modifications are members of the DNA methyltransferase (DNMT) family of enzymes. DNMT1 is the most abundant methyltransferase in somatic cells (Bender et al., 1999); it localizes to replication foci (Leonhardt et al., 1992) and interacts with the proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997). When a new DNA strand is synthesised, the methyl-CpG site is copied to an antisense CpG on the other strand, creating a hemi-methylated site. DNMT1 specifically recognises these hemi-methylated CpG (Pradhan et al., 1999) and transfers a methyl group to the unmethylated cytosine ring; in this way, methylation can be transmitted to both daughter cells. These features explain the stability of the modification, which enables it to be inherited in cell division. DNMT1 is necessary for correct embryonic development, imprinting and X-inactivation (Beard et al., 1995; Li et al., 1993; Li et al., 1992). DNMT3A and DNMT3B are required for the wave of *de novo* methylation that occurs in the genome following embryo implantation and of newly integrated retroviral sequences in mESC (Okano et al., 1999). Moreover, *Dnmt3a* and *Dnmt3b* knockout (KO) mice show severe developmental defects that cause death at the newborn and early embryonic stages, respectively. *DNMT3B* mutations underlie ICF syndrome, a rare disorder characterised by immunodeficiency, centromeric instability, and facial abnormalities (Hansen et al., 1999). These observations, coupled with *in vitro* data indicating that the DNMT3 enzymes show equal preference for hemi- and unmethylated DNA substrates, have led to the term "*de novo* methyltransferases" to describe DNMT3 (Okano et al., 1999). The fourth member of the family, DNMT2, has to date no clearly ascribed function in DNA methylation (Jeltsch et al., 2006), but transfer RNA methyltransferase activity has been reported (Goll et al., 2006).

The "eraser" responsible for reversion of this modification has not yet been identified univocally in animals (although it is described for plants (Choi et al., 2002)); some candidates have nonetheless been proposed (Kangaspeska et al., 2008; Ma et al., 2009; Metivier et al., 2008). Although there are numerous exceptions, most genome methylation is not maintained during meiosis and gamete formation (Abdalla et al., 2009). The entire genome undergoes global demethylation in gametes, and methylation is subsequently re-established in early embryonic

stages (Bestor, 2000) (see section on Epigenetics of pre-implantation development).

DNA methylation is linked to transcriptional silencing. Early experiments showed that DNA methylation leads to formation of a compacted, nuclease-resistant chromatin state (Keshet et al., 1986). It was proposed that the methyl group could interfere with protein binding to its cognate DNA sequence, such as a transcription factor (Bell and Felsenfeld, 2000; Hark et al., 2000; Holmgren et al., 2001; Tate and Bird, 1993). In most cases, however, methyl-CpG attracts rather than repels proteins; the "readers" for this modification are a family of proteins that contain the methyl-binding domain (MBD). These proteins link the CpG methyl group to complex chromatin remodelling machinery that turns off transcription and locks the chromatin in a condensed state (Hendrich and Bird, 1998). Methyl-CpG binding protein 2 (MECP2) was the first to be described (Lewis et al., 1992), and later gained in interest, as it is mutated in individuals with Rett syndrome (Wan et al., 1999). MECP2 has two domains: the MBD recognizes a symmetrically methylated-CpG dinucleotide (Wakefield et al., 1999), and a transcriptional repression domain interacts with several other regulatory proteins with HDAC activity (Jones et al., 1998; Nan et al., 1998). Other MBD-containing proteins have also been described, MBD1, MBD2, MBD3 and MBD4 (Ballestar and Esteller, 2002). Except for MBD4, which is involved in repairing DNA mismatches, the MBD family members belong to similar HDAC-containing complexes. All MBD proteins interact specifically with methylated DNA except MBD3 (targeted to methylated DNA through association with MBD2). An unrelated protein, Kaiso, also binds methylated DNA and interacts with a HDAC complex, N-CoR (Yoon et al., 2003). Other levels of crosstalk between DNA methylation and histone modifications have been described: DNMT and MBD proteins can recruit the H3K9 methylase SUV3-9h1 (Fuks et al., 2003a; Fuks et al., 2003b); DNMT also interact with KMT such as EZH2 in the PRC2 complex, as described above, and with HDAC complexes (Fuks et al., 2000; Rountree et al., 2000).

DNA methylation and cancer

Cancer development is thought to proceed via an evolutive process, in which a succession of proliferatively advantageous genetic changes, including mutations in tumour suppressor genes (TSG) and oncogenes as well as chromosome abnormalities, leads to the progressive conversion of normal cells into tumour cells (Aaltonen et al., 1993; Hahn et al., 1999; Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 1997). Epigenetic changes based mainly on the silencing of key regulatory genes are also implicated extensively in cancer development.

Global hypomethylation in cancer. Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells (Feinberg and Vogelstein, 1983); it is caused mainly by hypomethylation of repetitive DNA sequences, of coding regions and introns. This hypomethylation provokes activation of aberrant mitotic recombination, leading to chromosome rearrangements (Eden et al., 2003; Karpf and Matsui, 2005), reactivation of intragenomic endoparasitic DNA such as L1 and Alu repeats (Bestor, 2005), and disruption of genomic imprinting.

Hypermethylation of tumour suppressor genes in cancer. Methylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and is a mechanism for inactivation of TSG in addition to mutation (Baylin and Ohm, 2006; Esteller, 2008; Feinberg, 2004; Jones and Baylin, 2007). Nearly 50% of the genes that cause familial forms of cancer when mutated in germ-line are known to undergo methylation-associated silencing in various sporadic forms of cancer (Jones and Baylin, 2002). A long list of hypermethylated genes has now been obtained from various human neoplasias, converting this modification in a common hallmark of all types of human cancer (Esteller, 2007). Examples of genes with this aberrant methylation include p16INK4a, p14ARF, p15INK4b, adenomatous polyposis coli (APC), death-associated protein (DAP) kinase, p73, E-cadherin, von Hippel-Lindau (VHL), retinoblastoma (Rb), LKB1/STK11 and the oestrogen and progesterone receptors (Esteller, 2007). The importance of epigenetic gene silencing in cancer is also highlighted by the growing awareness that such changes can predispose to mutational events observed after promoter hypermethylation of DNA repair genes such as MLH1, BRCA1, MGMT, and the gene associated with Werner`s syndrome (WRN) (Herman and Baylin, 2003; Jacinto and Esteller, 2007a; Jacinto and Esteller, 2007b). It was suggested that the tumour-specific targeting of DNA methylation is pre-programmed by EZH2-containing PRC2, which normally has a role in marking embryonic genes for repression (Ohm et al., 2007; Schlesinger et al., 2007; Viré et al., 2006). The EZH2 system nonetheless does not contribute to *de novo* methylation of all tumour suppressor genes in cancer, since several tumour suppressor genes were not enriched with trimethyl-H3K27 when tested in normal cells. It thus appears that both gene targeting and adaptive mechanisms are involved

in *de novo* methylation in cancer (Esteller, 2008).

Sirtuins

Class III histone deacetylases are also termed sirtuins because of their homology to *Saccharomyces cerevisiae* Sir2, an enzyme implicated in epigenetic silencing of telomeres, ribosomal DNA and mating-type genes (Guarente, 1999). Sirtuins require NAD^+ as a metabolic cofactor to exert their enzymatic activity. The catalytic domain, highly conserved from Archea to man, can carry out two types of reactions: deacetylation and ADP ribosylation. In deacetylation, NAD^+ is hydrolyzed to generate nicotinamide, 2'-O-acetyl-ADP-ribose and a deacetylated target, whilst in ADP ribosylation, ADP-ribose binds to target protein and nicotinamide is released. NAD^+ is involved in the transfer of electrons generated through intermediary metabolism pathways; the NAD^+/NADH ratio is therefore an important sensor of the redox state of the cell and of metabolism. Two feedback mechanisms fine-tune the catalytic activity of sirtuins; nicotinamide is an enzyme inhibitor, as it competes directly with NAD^+ for binding to the catalytic domain (Landry et al., 2000), and NAD^+ levels control the activity of these enzymes (Sandmeier et al., 2002). The NAD^+/NADH ratio is itself regulated by other enzymes such as nicotinamide phosphoribosyl transferase (NAMPT), which uses nicotinamide to regenerate NAD^+, thereby promoting sirtuin activity (Yang et al., 2006). Sir2-compacted chromatin is characterised by hypoacetylation of lysine residues in the N-terminal tails of histones H3 and H4 (Braunstein et al., 1993); a very distinctive mark, hypoacetylation of H4K16, is a signature of Sir2 silencing (Guarente, 1999; Robyr et al., 2002; Suka et al., 2001). In addition to epigenetic silencing, Sir2 has a role in DNA repair, recombination, and DNA replication (Blander and Guarente, 2004; Gartenberg, 2000; Guarente, 1999). A very interesting role ascribed to Sir2 involves increased replicative lifespan, meaning the number of cell divisions that a mother cell can engage in during its life (Guarente, 1999). In the worm *Caenorhabditis elegans*, in *D. melanogaster* and possibly in mammals, Sir2 family members are also involved in lifespan (Baur et al., 2006a; Tissenbaum and Guarente, 2001). One proposed mechanism to explain the relationship between Sir2 and lifespan is caloric restriction (CR), which is known to increase lifespan in organisms from yeast to mammals (Cohen et al., 2004b; Guarente, 1999). CR produces a more oxidative metabolic state, reflected by high levels of NAD^+. This in turn activates sirtuins and facilitates survival mechanisms such as inhibition of senescence, activation of stress response pathways like FOXO-dependent signalling, and inhibition of apoptosis. Levels of one mammalian Sir2 homologue, SirT1, are increased under CR conditions in rats (Cohen et al., 2004b), although other studies postulate that CR-induced lifespan might be independent of these enzymes (Kaeberlein et al., 2005; Tsuchiya et al., 2006).

Mammals have seven sirtuin family proteins, termed SirT1 to SirT7. *In vivo*, SirT1, -2 and -3 behave preferentially as protein deacetylases, whereas SirT4 and -6 appear to have mono-ADP-ribosyl-transferase activity (SirT5 and -7 activity have not yet been fully carachterised) (Saunders and Verdin, 2007; Vaquero et al., 2007b). SirT1, -2, -3 and -6 have high activity on histone proteins, in particular on H4K16 and H3K9 residues (Vaquero et al., 2004). These are the only enzymes known thus far to deacetylate acetyl-H4K16 in higher eukaryotes (Michan and Sinclair, 2007; Vaquero et al., 2007b). Sirtuins (except for SirT6) are also implicated extensively in modification of non-histone proteins (Saunders and Verdin, 2007). SirT1 and -6 appear to be mainly nuclear, whereas SirT2 is preferentially cytoplasmic; SirT3, -4 and -5 are mitochondrial, and SirT7 is nucleolar (Michishita et al., 2005). In any case, none of these localisation is completely fixed, as various sirtuins can be translocated from one cell compartment to another in response to external or internal stimuli, developmental stage or other factors (Saunders and Verdin, 2007; Yamamoto et al., 2007).

SirT1

SirT1 is without doubt the most interesting mammalian sirtuin to date, with a staggering variety of functions, substrates, interactions and regulatory processes. It is a 747 amino acid protein of ~80 kDa and is expressed in all organs, although higher levels tend to be found in energy-dependent tissues (Michishita et al., 2005).

SirT1 functions

SirT1 has been implicated in processes as varied as metabolism, differentiation, cancer and HIV infection (Fig I-6). Beginning with evidence at the organism level, several groups have used targeted gene disruption to study SirT1 function at the organism level. SirT1 mutant mice generated by various targeting strategies have distinct phenotypes. SirT1 KO mice in R1 mESC (derived from 129Sv × 129Sv-CP embryos), carrying a truncation mutation through replacement of exons 5 and 6 with a hygromycin gene, show prenatal or perinatal death

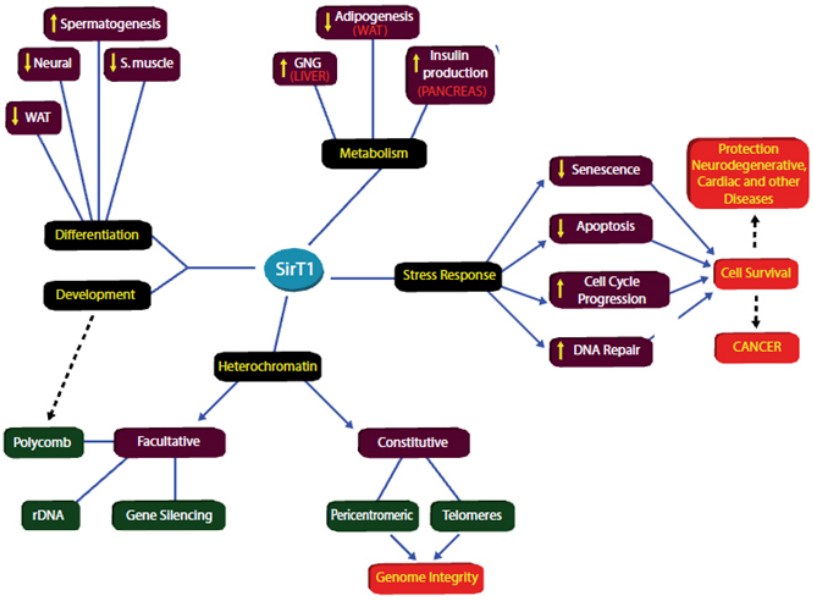


Figure I-6. SirT1 functions (adapted from Vaquero, 2009).
WAT, white adipose tissue; GNG, glucagon

in half of individuals; the remainder are smaller but survive to adulthood, suffer defects in gametogenesis and sterility, eyelid-opening problems, chronic lung infection and pancreatic atrophy (McBurney et al., 2003b). No global defects were detected in gene silencing in these mutant mice (McBurney et al., 2003a). Another KO was generated in TC1 mESC (129SvEv/C57BL/6 background) by deleting exon 4. Up to 90% of these mutants died perinatally, with developmental defects of the retina and heart, and the remaining 10% survived to weaning (Cheng et al., 2003). As cells from this KO showed p53 hyperacetylation following DNA damage and increased ionizing radiation-induced apoptosis in thymocytes, SirT1 deficiency was suspected to activate p53, leading to lethality in mutant mice. A more severe mutation was generated from TC1 cells by deleting exons 5 and 6 (as no truncated protein is detected, it is considered a null mutation), which led to death of all homozygotic mouse embryos by embryonic day E17.5-E18.5 (Wang et al., 2008). Mutant embryos analysed at E10.5–E12.5 showed an altered pattern of histone modification, i.e., reduced levels of trimethyl-K9 and increased acetylation of H3K9, reduced chromosome condensation, and loosely compacted chromosomes in metaphase. This chromatin phenotype provoked formation of chromosome bridges, chromosome breaks, unequal chromosome segregation, and aneuploidy, accompanied by impaired microhomology-mediated DNA damage repair and double-strand break (DSB) repair in SirT1 mutant cells.

Overall, this puzzling variety of results suggests that SirT1 acts in early development, in a role that is not likely to be essential, since mice pass through early embryonic stages and in some cases reach adulthood; SirT1 might have a modulatory effect on basic developmental processes, apparently dependent on genetic background. Mice with moderate overexpression of Sirt1 (a two- to four-fold increase) were also recently generated (SuperSirt1); they bear a single complete copy of the *Sirt1* gene in its natural genomic context. These mice are phenotypically indistinguishable from wt mice, indicating that SirT1 overexpression in development is probably compensated in the long term throughout this process. Differences have been detected at the metabolic level; these include increased energy expenditure accompanied by increased food intake and, on a high-fat diet, lower lipid-induced inflammation, improved glucose tolerance, and protection from hepatic steatosis (Pfluger et al., 2008).

SirT1 and metabolic regulation. As demonstrated by the phenotype of the SuperSirt1 mouse and based on numerous in vitro data, SirT1 has a preponderant function in metabolism processes. SirT1 is upregulated in fasting conditions. It appears to be involved in two important metabolic mechanisms: regulation of the activity of certain metabolic enzymes and regulation of endocrine signalling. For example, SirT1 deacetylases AceCS1, the cytosolic isoform of acetyl-CoA synthetase involved in fatty-acid formation from acetate (Hallows et al., 2006), enhancing its activity. SirT1 also promotes insulin production in pancreatic βcells (Bordone et al., 2006; Moynihan et al., 2005), hepatic gluconeogenesis through interaction and deacetylation of the transcription regulator PGC1α (Rodgers et al., 2005), and it inhibits adipogenesis through its action on the transcriptional activator peroxisome proliferator-activated receptor γ (PPARγ) (Picard et al., 2004).

SirT1 and cell survival. SirT1 is a key enzyme in the

regulation of cell survival, especially in oxidative and genotoxic stress conditions (Giannakou and Partridge, 2004; Haigis and Guarente, 2006). Through modulating processes such as DNA damage sensing, DNA repair and detoxifying machinery induction, inhibition of apoptosis and of senescence, cell proliferation, and autophagy stimulation, SirT1 mediates cell survival and rescue processes from tissue degeneration. SirT1 deacetylates FOXO family proteins (Brunet et al., 2004; Motta et al., 2004; Van Der Horst et al., 2004) and NFκB (Yeung et al., 2004), thereby promoting transcriptional activation of DNA repair and DNA detoxifying machinery, and repressing cell cycle control genes and apoptosis induction mediated by these transcription factors.

SirT1 also deacetylates p53, inhibiting p53-dependent apoptosis and senescence (Luo et al., 2001; Vaziri et al., 2001). SirT1$^{-/-}$ mouse embryonic fibroblasts (MEF) show p53 hyperacetylation (Cheng et al., 2003), and an inhibitory effect on p53 translocation to mitochondria after stress has been reported in mES (Han et al., 2008).

SirT1 also interacts and targets DNA repair machinery. Its binding and deacetylation of NBS1, a checkpoint protein involved in DNA damage sensing and induction of DNA repair (Yuan et al., 2007), induces DNA repair and cell survival. SirT1 binds to and deacetylates Ku70, involved in DSB repair through non-homologous end joining (NHEJ), thereby promoting its binding to the pro-apoptotic factor Bax, sequestering it and inhibiting apoptosis induction (Cohen et al., 2004a). After DSB, SirT1 is recruited to the damage site, an event that correlates with chromatin compaction and silencing in the same region (O'Hagan et al., 2008). This recruitment seems to be associated with SirT1 relocalisation from its native site, which induces a change in the gene expression pattern that resembles mammalian aging (Oberdoerffer et al., 2008). Nonetheless, the effective role of SirT1 in the DNA repair process is unknown.

SirT1 regulates certain major players in cell cycle control, such as Rb and E2F1. Rb acetylation inhibits its phosphorylation-dependent binding to E2F, a key event in the cell cycle; this suggests that SirT1-mediated Rb deacetylation promotes its phosphorylation and cell proliferation (Wong and Weber, 2007). SirT1 also binds and deacetylates E2F1, inhibiting its pro-apoptotic activity and inducing cell proliferation (Wang et al., 2006).

SirT1 and chromatin regulation. The main biological form of SirT1 is a homotrimer complex of ~350 kDa (Vaquero et al., 2004), but in stem cells it forms the unusual PcG-related complex PRC4, functionally similar to PRC2 (Kuzmichev et al., 2005). Like all sirtuins, SirT1 lacks the ability to bind directly DNA and must be recruited to the target DNA regions, although SirT1-targeted genes are only beginning to be identified (Lara et al., 2009).

A model for SirT1 action in chromatin remodelling postulates that, following the arrival of SirT1 to chromatin, it deacetylates H4K16 and H3K9, and recruits histone H1 directly, which it deacetylates at K26 (Vaquero et al., 2004) (Fig. I-7); H1K26

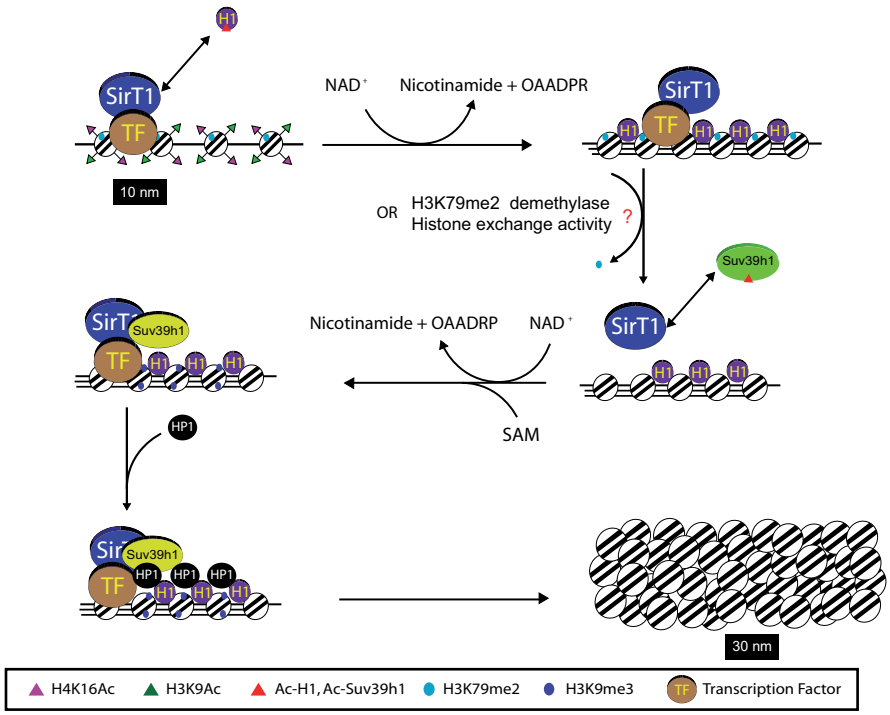


Figure I-7. Model of SirT1-linked heterochromatin formation. After recruitment by a TF, SirT1 promotes histone de-acetylation, particularly of H4K16 and H3K9, as well as recruitment and deacetylation of H1. This arrival coincides with loss of the active mark dimethyl-H3K79. Next, SirT1 recruits and de-acetylates Suv39h1, increasing its activity and methylates neighbouring nucleosomes. This in turn attracts HP1 and triggers heterochromatin spread.
Adapted from Vaquero, 2009.

can be then methylated by EZH2 (Kuzmichev et al., 2004), which coexists with SirT1 in the PRC4 complex. Thereafter, HP1 protein binds to dimethylated H1K26 through the HP1 chromodomain (Daujat et al., 2005; Nielsen et al., 2001). At the same time, the active chromatin mark dimethyl-H3K79 is lost, over a stretch of a few kilobases from the promoter regions (Feng et al., 2002; Kouskouti and Talianidis, 2005). Lastly, SirT1 promotes the establishment of heterochromatin marks, specifically trimethyl-H3K9 and methyl-H4K20, in the former case by interaction with Suv3-9h1 (Vaquero et al., 2007a). SirT1 induces Suv3-9h1-dependent H3K9 trimethylation by deacetylation of H3K9 and recruitment of Suv3-9h1 through its N-terminal region. Moreover, its binding to Suv3-9h1 increases its methyltransferase activity *in vitro* and *in vivo*, probably through a conformational change. Finally, SirT1 deacetylates K266 of Suv3-9h1, a residue located in the catalytic SET domain, rendering the enzyme more active.

SirT1 and its interplay with Suv3-9h1 and EZH2 could be also involved in DNA methylation. Both Suv3-9h1 and EZH2 bind directly to DNMT1 –and to DNMT3A in the case of Suv3-9h1– and direct DNA methylation to specific genes (Fuks et al., 2003a; Viré et al., 2006). SirT1 associates with the promoters of aberrantly silenced TSG in cancer cells, and SirT1 inhibition leads to their re-expression (Pruitt et al., 2006). In an experimental model in which a DNA DSB was induced in a reporter construct containing the E-cadherin promoter, SirT1 was found to bind to the region surrounding the DNA break in the reporter construct; this binding is necessary for DNMT3B recruitment and the subsequent heritable DNA methylation of the reporter gene promoter (O'Hagan et al., 2008).

Finally, SirT1 is also involved in the degradation of the H2A variant H2A.Z, which is associated with active chromatin and is essential in development. Deacetylation of H2A.Z by SirT1 in condition of cardiac hypertrophy induces cell growth and inhibits apoptosis (Chen et al., 2006a).

SirT1 in development and differentiation. , SirT1 levels are high in mESC and decrease after differentiation (Kuzmichev et al., 2005), indicating an antagonistic relationship of SirT1 in differentiation. Evidence shows that SirT1 function is important in two of the most metabolically-dependent tissue types, skeletal muscle and adipose tissue. Upon fasting, SirT1 upregulation induces silencing of certain key genes in both tissues to inhibit differentiation. In skeletal muscle, myogenin and myosin heavy chain (MHC) genes are silenced by SirT1 through the binding and deacetylation of transcription factor MyoD and the HAT PCAF (p300/CBP associating factor) (Fulco et al., 2003). In adipose tissue, SirT1 inhibits activation of genes such as fatty acid-binding protein (aP2) through recruitment of the corepressors NCoR and SMRT to the PPARγ-response genes, resulting in mobilisation of fat as well as inhibition of differentiation (Picard et al., 2004). Recent data suggest that differentiation in nerve tissue is likewise dependent on metabolic changes and regulated by SirT1. Under oxidative stress, mouse neural precursor cells (NPC) stop proliferating and differentiate into astroglial cells (rather than neurons) through a SirT1-dependent mechanism (Prozorovski et al., 2008). This mechanism relies on modulation of Hes1 by SirT1, which induces silencing of the pro-neuronal gene Mash1.

Regulation of Sirt1 expression

SirT1 expression is regulated in a complex manner at the transcriptional as well as at the post-transcriptional level.

SirT1 at the transcriptional level. In conditions of oxidative stress, two transcription factors have been identified that modulate SirT1 expression: E2F1 and HIC1 (Chen et al., 2005; Wang et al., 2006). Oxidative stress is known to stabilize E2F1, which binds directly to the *SIRT1* promoter at position -65 bp, leading to increased *SIRT1* transcription (Wang et al., 2006). Conversely, HIC1 (hypermethylated in cancer 1) is a negative regulator of SirT1 expression (Chen et al., 2005), binding to positions at −1116 bp and −1039 bp within the *SIRT1* promoter. Furthermore, p53 binds to two sites within the *SIRT1* promoter (−178 bp and −168 bp), repressing SirT1 expression (Nemoto et al., 2004). In the absence of nutrients, *SIRT1* transcription is induced through nuclear translocation of FOXO3a, which interacts with p53, thereby inhibiting p53 suppressive activity.

SirT1 at the mRNA level. HuR, a ubiquitously expressed RNA binding protein (Brennan and Steitz, 2001), associates with the 3′ untranslated region of SirT1 mRNA in physiological conditions (Abdelmohsen et al., 2007), leading to increased SirT1 mRNA stability and elevated protein levels. By contrast, the complex of HuR with SirT1 mRNA is disrupted by oxidative stress, leading to SirT1 downregulation. HuR binding to SirT1 mRNA is itself regulated by checkpoint kinase 2 (CHK2)-mediated phosphorylation, which in turn is activated through oxidative stress. This explains how HuR binding affinity to SirT1 transcript is reduced in these conditions. Methylation at Arg217 by the arginine methyltransferase CARM1 also

regulates HuR transcript-stabilising activity; LPS treatment of mouse neutrophils leads to HuR methylation-mediated stabilisation of TNFα transcript (Li et al., 2002a). CARM1-mediated methylation also regulates a HuR homolog, HuD, in this case by inhibiting p21cip1/waf1 mRNA stabilization in PC12 cells (Fujiwara et al., 2006).

MicroRNA (miR) are short 18–24 nt RNA that inhibit transcription or translation of mRNA. In human cells, miR34a binds the 3' UTR of the SirT1 mature transcript and inhibits its translation. miR34a inhibition of SirT1 leads to an increase in acetylated p53 and to expression of p21 and PUMA, transcriptional targets of p53 that regulate the cell cycle and apoptosis, ultimately provoking apoptosis in colon cancer cells (Yamakuchi et al., 2008).

SirT1 at the protein level. Phosphorylation is another important mechanism of SirT1 regulation. Large-scale mass spectrometry studies on HeLa nuclear cell extracts identified Ser27 and Ser47 as potential phosphorylation sites located within the SirT1 N-terminal domain (Beausoleil et al., 2004; Beausoleil et al., 2006). JNK2-dependent phosphorylation of Ser27 was first demonstrated to greatly affect SirT1 protein stability in cancer cells, and is the main element responsible for SirT1 overexpression in certain cancers (Ford et al., 2008). CyclinB/Cdk1 was later shown to phosphorylate SirT1, possibly in two residues (Thr530 and Ser540), enhancing its activity (Sasaki et al., 2008). Ser659 and Ser661 were identified as casein kinase 2 (CK2) phosphorylation sites (Zschoernig and Mahlknecht, 2009). CK2 also phosphorylates the conserved residues Ser154, Ser649, Ser651 and Ser683 of mouse SirT1, increasing its deacetylation rate and substrate-binding affinity. CK2-mediated phosphorylation increases the ability of SirT1 to deacetylate p53 and to protect cells from apoptosis after DNA damage (Kang et al., 2009a). cJUN N-terminal kinase (JNK1) was identified as a SirT1 kinase in conditions of oxidative stress; it requires phosphorylation-mediated activation of JNK1 and affects SirT1 Ser27, Ser47, and Thr530. The effect of these modifications is to induce SirT1 nuclear localization and enhance its enzymatic activity selectively on histone H3, but not on p53 (Nasrin et al., 2009).

SirT1 protein is also sumoylated on lysine residues. Sumoylation has regulatory effects on its substrates, such as alteration of subcellular localization, protein–protein interactions and enzymatic activity (Verger et al., 2003). Desumoylation is catalysed by a family of specific isopeptidases called the SENP desumoylases (Yeh et al., 2000). SirT1 is sumoylated at Lys734 in the normal state, whereas genotoxic stress leads to SENP1-driven desumoylation of SirT1, impairing deacetylase activity and thereby promoting activation of its apoptotic substrates and inducing cell death (Yang et al., 2007).

Subcellular localization is an additional regulatory tool for SirT1 activity. SirT1 is indeed subject to nucleocytoplasmic shuttling during oxidative stress (Jin et al., 2007; Tanno et al., 2007); in these conditions, cytoplasmic accumulation of SirT1 sequesters it from its nuclear substrates, rendering it unable to inactivate its predominantly anti-apoptotic substrates. Consequently, cytoplasmic localization of SirT1 appears to sensitize cells to oxidative stress-mediated apoptosis. SirT1 localisation is also implicated in differentiation of neural precursor cells (NPC); SirT1 was found in cytoplasm of embryonic and adult NPC and in response to differentiation stimuli, localised transiently in the nucleus, where it induces neuron differentiation (Hisahara et al., 2008).

SirT1 regulation via protein-protein interactions can be mediated by distinct proteins. AROS (active regulator of SirT1) is a positive regulator of SirT1, increasing its deacetylase activity (Kim et al., 2007), whereas DBC1 (deletions in tumour suppressor breast cancer 1) acts as a SirT1 inhibitor, binding its catalytic pocket (Kim et al., 2008b).

Epigenetic mechanisms in embryonic stem cell differentiation

Early in development, the first zygote-derived cells must perform two different functions. One is self-renewal, which is the ability to expand the cell population that maintains a certain degree of potency until a definite developmental stage is reached. The other function is totipotency (for the zygote and first cell divisions; pluripotency for the ICM and derived cultured ES cells), the ability to maintain the potential to give rise to any differentiated cell of the adult body. To achieve totipotency, germ cell epigenetic states must first be reset, and the zygotic genome must then be activated to allow expression of appropriate genes in subsequent development (Morgan et al., 2005; Reik, 2007). When a differentiation step is to be initiated, each cell must be able to modify its gene expression programme to change its phenotype. To simplify a complex scenario that has only begun to be described, a stem cell appears to have a globally open chromatin structure that keeps pluripotency-maintenance genes active and differentiation genes silenced, in a reversible, highly plastic way. Following differentiation

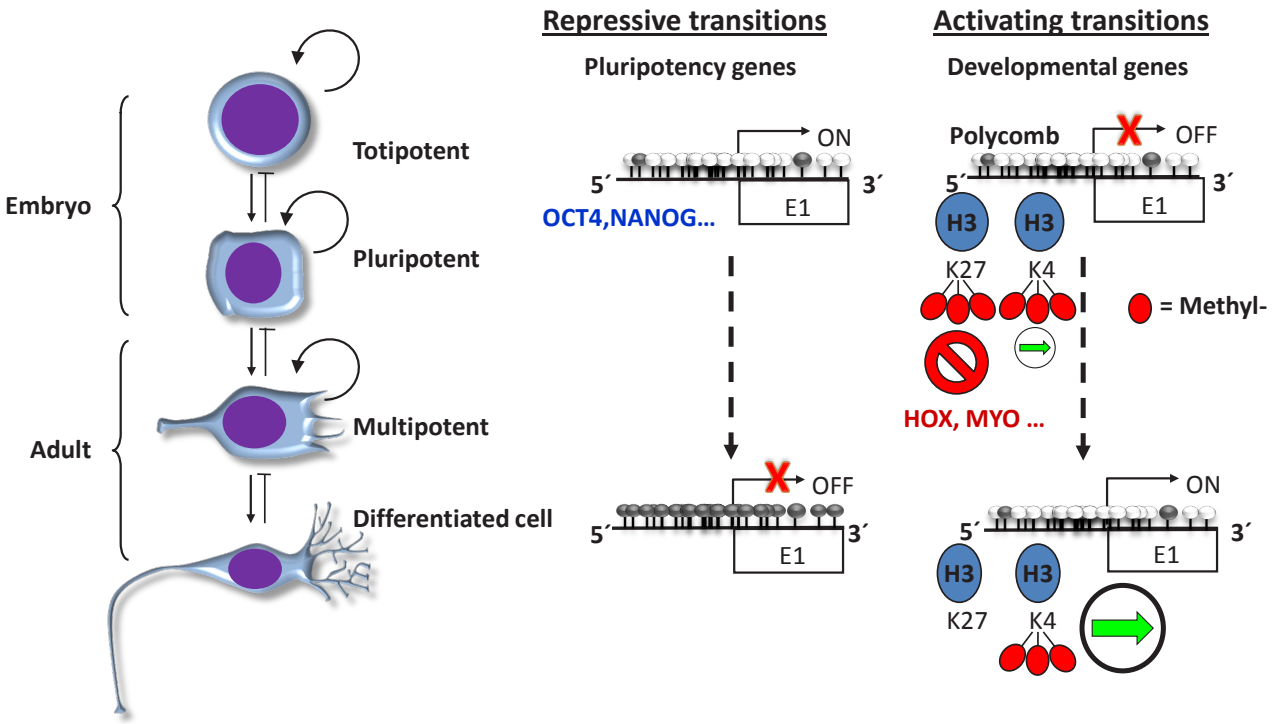


Figure I-8. Epigenetic regulation of hESC differentiation. hESC differentiation involves inactivation of pluripotency genes (repressive transitions) and activation of developmental genes (activating transitions). Many pluripotency genes are inactivated during differentiation through promoter hypermethylation. The best-known mechanism involved in developmental gene silencing in hESC is bivalent mark/Polycomb-mediated inactivation, whose resolution is responsible for developmental gene activation during differentiation.

towards a definite lineage, a stem cell must a) downregulate pluripotency genes definitively, b) activate expression of genes for the necessary differentiation phenotype, and (Fig. I-8) c) lock differentiation genes for other lineages into a silent state. Moreover, these processes must be precisely regulated in space and time. All of these skills are conferred on a stem cell mainly by the action of the epigenetic machinery.

Epigenetic events in pre-implantation phases. Two cycles of DNA methylation characterise gametogenesis and pre-implantation development in mammals (Fig. I-9). In the first week of embryonic development, primordial germ cells arise in the extraembryonic region posterior to the primitive streak, and are designated to regain the capacity to form a new organism (Hayashi et al., 2007; Saitou et al., 2002). Primordial germ cell differentiation involves a wave of genomic demethylation, which resets differentially methylated loci, including imprinted and allele-specific regions, followed a few days later by methylation (Lees-Murdock and Walsh, 2008). Mature sperm and oocytes remain highly methylated until fertilization, after which a second cycle of demethylation and gradual

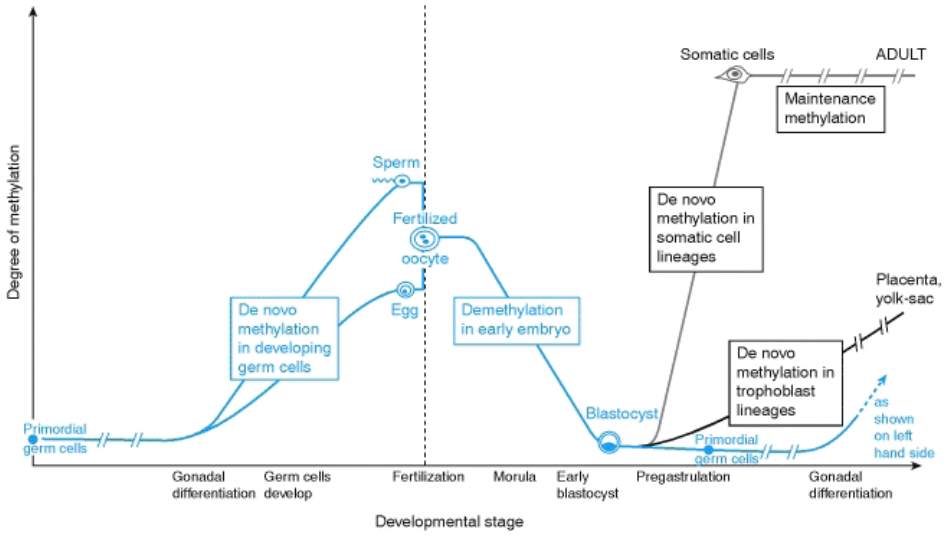


Figure I-9. Changes in DNA methylation during mammalian development. Note the very rapid changes in DNA methylation during: (i) gametogenesis - de novo methylation gives rise to substantially methylated genomes in the sperm and egg (albeit with differences in both the overall level of methylation and the pattern of methylation in these genomes), and in (ii) in the early embryo, a wave of genome-wide demethylation occurs at the preimplantation stage (morula and early blastula), and is succeeded shortly afterwards by large-scale de novo methylation beginning at the pregastrulation stage. The latter is particularly pronounced in somatic lineages, and to a lesser extent in trophoblast lineages giving rise to placenta and yolk sac, but does not occur in the primordial germ cells (which will eventually give rise to sperm and egg cells).

re-methylation of parental genomes occurs. The paternal genome is rapidly demethylated by an active mechanism, since it occurs in the absence of replication or transcription (Mayer et al., 2000; Oswald et al., 2000). Various DNA repair enzymes have been proposed to carry out active demethylation in mammals (Gehring et al., 2009; Niehrs, 2009), and a role was recently suggested for the *de novo* methyltransferases DNMT3A and DNMT3B (Kangaspeska et al., 2008; Metivier et al., 2008). Coincident with the first embryonic cleavage divisions, the zygotic genome gradually becomes demethylated until the morula stage. This probably occurs through a passive mechanism, whereby newly replicated DNA strands fail to be methylated by DNMT1, resulting in an overall increase in unmethylated DNA (Rougier et al., 1998). This is due to sex-specific splicing events that control DNMT1 activity in early embryos by regulating its translation or nucleo-cytoplasmic localisation (Mertineit et al., 1998). *De novo* DNA methylation begins after blastocyst formation; cells that comprise the ICM show higher methylation levels than those of the trophectoderm (Santos et al., 2002), probably due to differential regulation of DNMT3B expression, decreased in extra-embryonic tissues and increased in ICM-derived cells (Hirasawa and Sasaki, 2009).

Histone modification also undergoes specific regulation during preimplatation development. Following fertilization, sperm chromatin is decondensed by exchange of arginine-rich protamines for histones, and becomes associated with hyperacetylated and hypomethylated H4 (Adenot et al., 1997). In contrast, oocyte chromatin displays abundant H3K4 methylation before and after fertilization (Lepikhov and Walter, 2004; Van Der Heijden et al., 2005). In the male pronucleus, H3K4 is monomethylated shortly after fertilization, but it is not until 8-10 hours post-fertilization that the trimethylated form is observed. Female pronuclear chromatin is methylated extensively on H3K9, a repressive mark, whereas only the monomethylated form is found in the male pronucleus (Arney et al., 2002; Cowell et al., 2002; Liu et al., 2004; Santos et al., 2005). Trimethyl-H3K27 is enriched in maternal, but not paternal chromatin following fertilization (Erhardt et al., 2003). By the four-cell stage, all blastomere nuclei show equivalent levels of methyl-H3K27, methyl-H3K4, and methyl-H3K9. Many developmental gene promoters were recently shown to be wrapped on histones rather than on protamines already in the mature sperm, and are pre-marked with distinctive chromatin domains. Dimethyl-H3K4 is enriched at certain developmental promoters and trimethyl-H3K4 localizes to a subset of these promoters, including regions in HOX clusters, ncRNA, and paternally expressed imprinted loci, while trimethyl-H3K27 is enriched at developmental promoters repressed in early embryos. Furthermore, developmental promoters are generally DNA hypomethylated in sperm, and are methylated during differentiation (Hammoud et al., 2009).

There is also a discrepancy in histone methylation levels between cells of the ICM and the trophectoderm. Pluripotent cells in the ICM show extensive H3K27 methylation, but in the trophectoderm, methyl-H3K27 is only detected on the inactive X chromosome. Substitution of canonical for variant histones also contributes to epigenetic regulation in preimplantation embryos (Santenard and Torres-Padilla, 2009).

Epigenetics of embryonic stem cells. ES cells are thought to be an adaptation of cell culture conditions, because the properties of the ICM are transient in nature, whereas cultured ESC divide indefinitely without losing pluripotency. Epigenetic changes during ESC differentiation in culture might nonetheless provide an informative model for events that regulate early embryonic development.

ES cells must retain pluripotency while proliferating rapidly in culture. A group of transcriptional regulatory proteins are critical for pluripotency maintenance of both ES cells and the ICM (Jaenisch and Young, 2008). The POU5F1 (OCT4) and NANOG transcription factors are necessary for correct ICM and ESC differentiation (Chambers et al., 2003; Nichols et al., 1998) and, in conjunction with the high-mobility-group SOX2 protein, directly control the expression of genes important for both pluripotency and developmental pathways (Boyer et al., 2005). This network activates genes needed for ESC survival and proliferation, while repressing target genes that are activated only during differentiation (Loh et al., 2006; Sharov et al., 2008). With increasing research into the ES transcriptional network, other factors are added continually to this core “trio”. . A notable addition is zinc finger transcription factor sal-like protein 4 (SALL4) (Wu et al., 2006; Yang et al., 2008; Zhang et al., 2006). Co-expression and coregulation of these factors is univocally associated to pluripotency. OCT4, NANOG, SOX2 and SALL4 are able to regulate their own expression and control a set of target genes through mutual heterodimerization and/or shared promoter occupancy, forming a self-reinforcing circuit of pluripotency (Chew et al., 2005). In mESC, Nanog and Oct4 are associated to each other, and recruit specific repression complexes to their target genes, including elements of the NuRD and Sin3A complexes. A previously undescribed

Hdac1/2- and Mta1/2-containing complex, defined as NODE (Nanog and Oct4-associated deacetylase), might be the main effector of gene repression mediated by these factors (Liang et al., 2008). OCT4 NANOG SOX2 and SALL4 also associate to other components of the epigenetic machinery such as the PRC1 components EED, SUZ12, or the HMT G9A (Loh et al., 2006; Ura et al., 2008). SALL4 has a specific role in pluripotency factor/epigenetic modifier crosstalk, as it binds cooperatively with PRC1 and PRC2 to trimethyl-H3K27 at some promoters, but does not require these repressive complexes for binding at other sites (Yang et al., 2008). Detailed analysis of the pluripotency network showed that Nanog plays the leader role for generation and maintenance of the ground state of pluripotency, both *in vivo* (in the epiblast) and *in vitro* (in hESC and iPS generation) (Silva et al., 2009).

In addition to regulating a network of developmentally important genes in ESC, OCT4 positively regulates expression of the di- and trimethyl-H3K9 demethylases KDM3A (JMJD1A) and KDM4C (JMJD2C) (Loh et al., 2007). Undifferentiated ES cells appear to have a more open chromatin structure than differentiated cells (Meshorer and Misteli, 2006; Meshorer et al., 2006) and are depleted of trimethyl-H3K9 (Meshorer et al., 2006). Activation of H3K9-specific demethylases by the core pluripotency regulatory machinery would cause an overall reduction in trimethyl-H3K9, directly linking the ES cell pluripotency circuitry to the formation of an open chromatin structure (Loh et al., 2007). In addition to post-translational chromatin modification, CHD1 remodelling of chromatin structure also performs its function by maintaining an open chromatin state in ES cells, localizing mainly in active gene promoters. Downregulation of this factor leads to heterochromatin accumulation and loss of pluripotency, as cells can no longer give rise to certain lineages (Gaspar-Maia et al., 2009).

As stated above, ES cells must maintain its differentiation genes silenced. This is achieved at least in part through the creation of bivalent chromatin domains. These domains are termed “bivalent”, as they contain overlapping regions of the transcriptionally permissive histone modification trimethyl-H3K4 and the transcription silencing mark trimethyl-H3K27, mediated respectively by the mixed-lineage leukaemia (MLL) complex and the EZH2 component of PRC2 (Bernstein et al., 2006; Schuettengruber et al., 2007). Bivalent domains maintain silencing of developmentally important genes –primarily transcription factors such as the *Hox* and other tissue-specific genes– while simultaneously keeping them poised for either repression or activation, depending on the developmental lineage for which the ESC are destined (Bernstein et al., 2006). In essence, this bivalent configuration is thought to enable retention of developmental plasticity by these genes.

Histone KDM are responsible for the resolution of bivalent chromatin domains (Cloos et al., 2008). For example, *Hox* gene expression is strongly upregulated following ES differentiation, and trimethyl-H3K27 demethylase KDM6A (formerly UTX) binding at HOX promoters is increased concomitantly; this leads to a reduction in di- and trimethyl-H3K27 and dissociation of PRC2 (Agger et al., 2008; Lan et al., 2007). Strikingly, Jarid2 (JMJ), a H3K27 demethylase, is physically associated to the PRC2 complex in stem cells, and is required for efficient PRC2 binding to its target, but also inhibits K27 methylation, thus acting as an internal modulator of PRC2 during development (Peng et al., 2009; Shen et al., 2009). Other bivalent domain-marked genes require stable repression within specific developmental pathways. This is achieved by the tandem action of PRC2 and the trimethyl-H3K4 demethylase KDM5A (also known as JARID1A), whose recruitment results in increased trimethyl-H3K27 and decreased trimethyl-H3K4 at these promoters (Pasini et al., 2008).

There are other important modifications implicated in the first stages of developing embryo. In the mouse embryo, H3R26 and R17 methylation levels are heterogeneously distributed as early as four-cell stage blastomeres; cells with higher levels of this mark are more likely to contribute to ICM, while lower levels target cells to mural trophoectoderm. This observation indicates that the only real totipotent cell might in fact be the zygote. CARM1, the enzyme responsible for these modifications, thus seems to be necessary for blastomere and ICM cell pluripotency; CARM1-overexpressing cells strongly upregulate Nanog and Sox2 (Torres-Padilla et al., 2007). CARM1 is also needed for ES cell self-renewal and pluripotency, as CARM1 knockdown downregulates pluripotency genes, leading to differentiation. CARM1 associates with *Pou5f1* and *Sox2* promoters, which have detectable levels of R17/26 histone H3 methylation (Wu et al., 2009).

Compared to differentiated cells and cancer cells, hESC possess a unique DNA methylation signature (Ball et al., 2009; Bibikova et al., 2006; Deng et al., 2009; Fouse et al., 2008; Meissner et al., 2008), supporting the concept that a specific DNA methylation pattern could contribute to the pluripotent state. A

genome-wide DNA methylation study in several somatic cell types and sperm was the first to highlight a relationship between promoter DNA methylation and promoter activity, which apparently depends on promoter CpG content (Weber et al., 2007). Promoters with low CpG levels showed no correlation between activity (defined by RNA polymerase II occupancy, not in itself an indicator of full transcriptional activity, especially in ES cells (Efroni et al., 2008; Guenther et al., 2007)) and methylated CpG abundance. Low CpG promoters (LCP) would be methylated regardless of their activity status, intermediate CpG promoter (ICP) activity would correlate inversely to the extent of methylation, and high CpG promoters (HCP) would only weak or no methylation, even when inactive. Genome-wide high-resolution DNA methylation profiling across promoter regions was later carried out in mESC, superimposing chromatin state maps (histone modifications) on a given DNA methylation "background" (Fouse et al., 2008); over 6100 genes that were hypermethylated over the promoter regions examined were annotated. DNA methylation in mESC occurs primarily in ICP and LCP, or in non-CpG island regions of HCP. Gene ontology analysis of the methylated genes showed that these genes encode sensory perception and signalling molecules, suggesting that methylated genes are involved mainly in late differentiation and signal transduction processes, and are not expressed in mESC, whereas unmethylated promoters associated with transcription, RNA and protein metabolic processes, cell survival and proliferation. This suggests that unmethylated promoters show reasonable correlation with genes that are active in ESC.

DNA methylation appears to be associated to specific histone marks (Meissner et al., 2008). For promoters occupied by histones methylated at H3K4 and/or H3K27, occupancy of promoter sequences by an activating histone mark (i.e., H3K4) correlates negatively with DNA methylation, while occupancy by a repressive histone mark (i.e., H3K27) is positively correlated with DNA methylation. Promoters that lack both histone marks are highly methylated, and DNA methylation status is a good predictor of gene expression. During cell differentiation, hypermethylation can occur at CpG island promoters and at CpG-rich sequences outside promoter regions (Meissner et al., 2008). Remarkably, the authors claim that almost no demethylation is detected, and focus their attention mainly on HCP; they suggest that DNA methylation-mediated epigenetic repression increases globally during lineage specification. Many of the identified targets of differentiation-coupled *de novo* DNA methylation are stem cell- and germline-specific gene promoters (Farthing et al., 2008; Mohn et al., 2008). One interpretation of this selectivity is that DNA methylation might stably repress the pluripotency program and prevent its aberrant reactivation and de-differentiation under physiological conditions. Experimental support for this model comes from a recent report showing that somatic cell reprogramming into iPS is greatly enhanced by treatment with the DNA methyltransferase inhibitor 5-azacytidine (Mikkelsen et al., 2008). DNA methylation profiling of iPS compared to the primary fibroblasts from which they were derived clearly shows a large group of genes that are demethylated upon reprogramming, related mainly to pluripotency (Doi et al., 2009). At the same time, many developmental genes are hypermethylated during reprogramming and correlate inversely with the presence of a bivalent mark. Genetic and molecular data are thus compatible with a role for DNA methylation in the shut-down of pluripotency and, eventually, cell specification.

A powerful single-base mapping method was recently used to define the hESC methylome (Lister et al., 2009). Around one-quarter of all methylation is in a non-CG context (mCHG and mCHH, where H = A, C or T), a phenomenon nearly undescribed in animals, although there were some reports (Ramsahoye et al., 2000; Woodcock et al., 1987). Methylation in nonCG contexts shows enrichment in gene bodies and depletion in protein-binding sites and enhancers; it disappears after induced hESC differentiation, and is restored in iPS. Hundreds of differentially methylated regions were also identified proximal to genes involved in pluripotency and differentiation (Lister et al., 2009).

In any case, the role of DNA methylation in lineage specification has not been elucidated exhaustively. Functional data demonstrate that treatment with the DNA demethylating drug 5-aza-2′-deoxycytidine greatly enhances cardiomyocyte differentiation from hES (Xu et al., 2002) or from the embryonic carcinoma cell line P19 (Choi et al., 2004), and endothelial differentiation from mES (Banerjee and Bacanamwo, 2010). Genes involved in this differentiation effect are still unidentified. Only a few genes with a LCP appear to be the only ones prone to promoter demethylation during differentiation. We thus studied this phenomenon further to identify a more defined function of promoter methylation in lineage specification.

OBJECTIVES

OBJECTIVES

The epigenetics of stem cells has been of central interest in biology in recent decades. Since human ESC became available to developmental and molecular biologists, and high-throughput techniques now provide the possibility of rapid, large-scale analysis of the epigenetic panorama, much effort has been dedicated to the detailed description of stem cell epigenetics. Our research focussed on two aspects of stem cell epigenetics: DNA methylation and histone modification by the histone deacetylase SirT1.

Embryonic stem cells have a unique epigenetic signature. Bibikova et al. described 23 genes whose promoter methylation status was unique to hESC; DNA methylation was also reported to stably inactivate pluripotency genes during hESC differentiation. Certain late differentiation genes, generally bearing a low density CpG promoter and not showing the bivalent mark, are frequently hypermethylated in stem cells. Nevertheless, developmental gene activation through promoter demethylation has not been fully investigated. We approached this point to specifically define genes methylated in hESC as compared to normal tissues. As promoter hypermethylation is a hallmark of many cancers, we also analysed similarities in gene promoters hypermethylated in stem cells and in cancer; this could aid in understanding the aberrant process of cancer promoter methylation, and because an important concern for stem cell therapeutic application is the potential for tumour transformation.

It has long been known, and was recently confirmed in undifferentiated hESC, that most developmental genes are repressed by Polycomb repressive complexes. A specific signature in hESC, the bivalent mark, is observed at the developmental promoters, mediated mainly by PRC2 and other complexes with histone methyltransferase activity. Although the role of histone methylation in developmental gene regulation has been characterised extensively, histone acetylation in this context is less well-studied. The histone deacetylase SirT1 was previously implicated in tissue differentiation processes, i.e. muscle, adipose tissue and neurons. SirT1 is highly expressed in stem cells and colocalises on chromatin with components of the PRC. SirT1-deficient (KO) mice show marked developmental defects, although in many cases they pass through early embryonic stages. We studied SirT1 in early hESC differentiation, to determine its role in modulating gene expression though chromatin modification.

The concrete objectives of this study were to:

1. Characterise the role of DNA methylation in human embryonic stem cell differentiation, with special attention to demethylation-mediated gene regulation.

2. Determine the role of SirT1/histone modification-mediated gene regulation during embryonic stem cell differentiation.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

Stem cell line culture, differentiation and treatment

Human stem cell lines. Cell pellets and/or DNA/RNA were obtained from the following laboratories: Shef-1 (Servicio de Inmunologia, HUCA, Oviedo, Spain), Shef-4, Shef-5, Shef-7, H7, H14 (Centre for Stem Cell Biology, University of Sheffield, Sheffield, UK), H181 (Centro Andaluz de Biología y Medicina Regenerativa, Seville, Spain), I3 (Institute of Reconstructive Neurobiology, University of Bonn, Germany); cells were cultured and passaged following protocols established by each laboratory. The laboratories involved in the establishment and maintenance of these cell lines are members of the European project ESTOOLS (LSHG-CT-2006-018739); participant laboratories in ESTOOLS use only hESC lines derived from IVF embryos that will not be transferred into the uterus. These embryos were donated for research in accordance with the legal requirements of the country of origin. All donors gave written informed consent. The cell lines were established from different embryos and were maintained under distinct conditions, thereby ensuring the independence of results for type of line and culture conditions.

Stem cell culture. MEF (passage 2, Stem Cell Technologies) were thawed and expanded for 3 or 4 further passages in MEF medium [DMEM/F12 1:1 with 15% foetal bovine serum (FBS; Sigma) 1X non-essential amino acid mix (NEAA; Lonza) 1X glutamine (Lonza), 0.1 mM β-mercaptoethanol (BME; Sigma)], treated for 3 h with 10 μg/mL mitomycin C (Sigma), washed and seeded (3.5 x 10^5 in a 25 cm^2 gelatin-coated flask); once attached overnight (o/n), hESC were seeded on this feeder layer. Shef1 was cultured in hESC medium [KO-DMEM, 20% KO serum replacement (Gibco), 1X NEAA, 1X glutamine, 0.1 mM BME, 4 ng/ml basic-FGF (Peprotech)], with daily medium changes. After four to five days, hESC colonies were treated with 1 mg/ml collagenase IV (Gibco) in KO-DMEM for 5-10 min, washed to remove collagenase solution before colony detachment, then mechanically disrupted with a pipette or sterile glass beads, and divided 1:3 to 1:4. Differentiated colonies normally appeared at a low rate and were scraped or aspirated before passaging or collecting. Cells for pellets or for further differentiation were collected by collagenase IV treatment until colony detachment, which was interrupted before the feeder layer was disrupted.

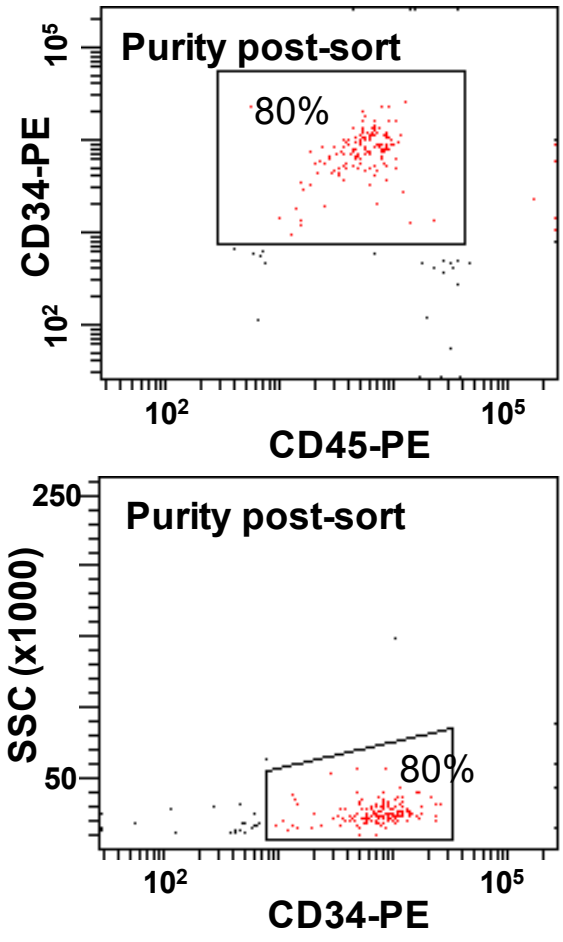
Stem cell differentiation. For differentiation of embryoid bodies (EB), hESC colonies were detached and incubated as floating aggregates for 15 days (or as indicated) in ultra-low attachment flasks (Corning) with hESC medium lacking bFGF. Medium was changed after 24 h to eliminate cell debris and apoptotic cells normally present the day after detachment; subsequently, medium was changed every 48 h. For further differentiation to fibroblast-like (F-L) cells (Li et al., 2009), 15-day EB were attached to gelatin-coated plates and cultured in DMEM with 15% FBS, 1X NEAA, and 1X glutamine for a further 15-20 days, passaging with trypsin two or three times. Cells finally appeared as a monolayer of fibroblasts and keratinocyte-like cells.

For *in vitro* neural differentiation (Pankratz et al., 2007), detached hESC colonies were cultured like EB for four days to initiate the differentiation process, then conditioned for an additional 2 days in neural medium (NM) [DMEM/F12, NEAA 1X, 2 μg/ml heparin for FGF stabilization, and the supplement N2 (Gibco)]. At day six, aggregates were attached on a laminin-treated substrate (20 μg/ml in culture medium, 37°C, 12 h) and were maintained in NM with 20 ng/ml bFGF. Attached aggregates flattened over 1-2 days, columnar cells developed and formed neural tube-like structures. At day 15, columnar cells in the centre of colonies were enzymatically separated with 1X dispase (Stem Cell Technologies) and grown as floating spheres for one more week in NM with bFGF, then reattached to a polyornithine/laminin substrate and cultured in neurobasal medium (Gibco), N2, 1X NEAA, supplemented with brain-derived neurotrophic factor (10 ng/ml; Peprotech), glial cell line-derived neurotrophic factor (10 ng/ml; R&D Systems), N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (1 μM; Sigma-Aldrich), ascorbic acid (200 M; Sigma-Aldrich), and laminin (20 μg/ml) until neurite processes were clearly observed (1-2 weeks). Marker expression for both differentiated cell types were confirmed by q-RT-PCR.

Mouse stem cells derivation, culture and differentiation. Mouse TC1 ES cells and the knockout ES line (SirT1$^{\Delta ex4/\Delta ex4}$) were a gift of Dr. Frederick Alt's laboratory (Cheng et al., 2003). Sirt1 transgenic ES cells were derived by the CNIO Transgenic Mouse Unit. Sirt1 transgenic (+/+;tg/.) and WT ES cells were established *de novo* from blastocysts obtained by *in vitro* fertilisation of WT C57BL/6 oocytes with sperm of a Sirt1 transgenic (+/+;tg/.) male (Pfluger et al., 2008) following standard protocols (Nagy et al., 2003). After fertilisation, embryos were cultured in KSOM (37ºC, 3 days) until they reached the blastocyst stage, then plated in ES cell medium with feeders until outgrowth of the inner cell mass was observed.

mESC medium is composed of high glucose DMEM with 15% FBS, LIF (1000 U/ml), 1X NEAA, Glutamax and BME. For EB differentiation, cells were trypsinised and seeded in the same medium without LIF; medium was every 24-48 h for 15 days.

Primary cells and tissues. Primary $CD34^+$ haematopoietic stem cells were purified from cord blood (CB) samples obtained from healthy newborns with maternal informed consent. CB harvesting procedures and informed consents were approved by the Local Hospital Ethics Board. Mononuclear cells were isolated using Ficoll-Hypaque (Amersham Biosciences). $CD34^+$ cells were purified by positive selection using anti-CD34 microbeads (Miltenyi Biotech). Immunomagnetic $CD34^+$ cell-containing cell suspensions were passed through Pro-MACS immunomagnetic columns (Miltenyi Biotech). The flow-through contained the purified $CD34^+$ fraction. The purity was 80% ± 12% ($n = 2$) (Figure I-10), as measured by flow cytometry (FACSCanto, Becton Dickinson) using a fluorochrome-conjugated anti-CD34 antibody (BD Biosceinces).



Fgure I-10. Flow cytometry analysis of CD34+ cell purity after positive selection using anti-CD34 microbeads. Detection signals were obtained using an anti-CD34 and an anti-CD45 antibody.

Lymphocytes and neutrophils from peripheral blood of healthy volunteers were separated by centrifugation, using Histopaque-1077 (Sigma). Lymphocyte-enriched fractions were obtained by collecting the upper cushion of mononuclear cells and granulocytes (mainly neutrophils) following haemolysis of the remaining pellet. RNA from breast, liver, heart, muscle, lung, colon, and lymph node samples were obtained from Ambion; DNA from breast, heart, brain, muscle, colon, brain and sperm was obtained from Biochain. Participating subjects were informed and gave written consent to all procedures.

Cancer cell lines. MDA-MB-231, Hela, CasKi, SiHa, HCC1937, BT-474, LoVo, HCT115, DLD1, Co115, HT29, SW48, HCT116, RKO (ATCC) were cultured in DMEM with 10% FBS, 1X NEAA, 1X glutamine, 1X penicillin-streptomycin (Gibco) at 37°C in a 5% CO2 incubator and were passaged by trypsinization when confluent. U937, HL60, AKATA, Raji, Ramos, Karpas, and Farage (ATCC) cell lines were maintained in the same conditions in RPMI medium with 10% FBS, 1X NEAA, 1X glutamine, 1X penicillin-streptomycin, and passaged by adding fresh medium, according to cell growth rate.

DNA methylation analysis

DNA methylation is a chemical modification in the 5 position of the cytosine aromatic ring, where a methyl group is added. It is generally found in a cytosine followed by a guanine at its 3' (with some exceptions described in the Introduction), and is thus symmetrically detectable on both DNA strands. CpG dinucleotides in the genome are concentrated in repetitive sequences and in regions spanning promoters and gene regulatory regions, called CpG islands. A CpG island is technically defined as a 200-bp window moving across a sequence of interest at 1-bp intervals, with a C + G content >50% and an observed/expected CpG frequency of >0.6 (Gardiner-Garden and Frommer, 1987), or a 500-bp moving window with a C + G content of >55% and an observed/expected CpG frequency of >0.65. The latter definition provides more accurate association of CpG islands with the 5′ region of genes and excludes most Alu repeats (Takai and Jones, 2002). As stated in the introduction, CpG island-containing promoters are further classified by some authors as HCP (high CpG promoter, which contains a 500 bp region with a GC content ≥0.55 and a CpG observed/expected ratio ≥0.6), LCP (low CpG promoter containing no 500 bp interval and with a CpG observed/expected ratio ≥0.4), and ICP (intermediate CpG content promoter with CpG density between HCP and ICP) (Mikkelsen et al., 2007; Weber et al., 2007).

Techniques used for CpG detection are always based on the differential recognition or the distinct chemical behaviour of a methylated vs. an unmethylated CpG. Differential recognition can be performed by a methylation-sensitive restriction enzyme (as in the amplification of intermethylated sites detailed below (Jorda et al., 2009)) or an antibody (methyl-DNA immunoprecipitation; meDIP (Jacinto et al., 2008)), whereas differential chemical reactivity is the principle of the bisulphite modification and the related techniques reported in the following sections.

DNA extraction. Extraction was performed with the Phase Lock Gel DNA extraction protocol (Eppendorf; for samples >5 x 10^6 cells) or with QIAamp DNA Blood Mini Kit (Qiagen; for small samples).

Amplification of intermethylated sequences (AIMS). Purified genomic DNA (1 μg) was digested sequentially with SmaI (Roche, 1U, 16 h, 25ºC) and XmaI (NEB; 5U, 6 h, 37ºC). Oligonucleotide adaptors Blue and MCA-Blue (see supplementary primer table) were annealed by heating (65ºC) and gradual cooling at RT for 60 min. Digested DNA was ligated with adaptors, 1 μmol/μg DNA, incubating with T4 DNA ligase (NEB 800U, 16 h, 16ºC). The ligation product was purified with GFX PCR DNA and Gel Band Purification Kit (Amersham) and amplified by PCR using the following primers (see primer table) and conditions: PCR primer Set1 CTG (denaturalisation 15 sec at 95ºC, annealing-extension 1 min at 74ºC, 29 cycles) and Set2 CTA (denaturalisation 15 sec at 95ºC, annealing 45 sec at 68ºC, extension 1 min at 72ºC, 30 cycles). PCR products were resolved in 6% polyacrylamide 8 M urea denaturing electrophoresis gels (4-5 h, 55W) and developed by silver staining. Indicated bands were extracted manually from the gel, and DNA resuspended in water, re-amplified with the corresponding primer and cloned in a pGEM-T easy vector (Promega). Positive plasmid clones were sequenced and blasted to the human genome in the NCBI Blast website.

Bisulphite modification. Genomic DNA was treated with sodium bisulphite (Sigma) either using the EZ-DNA methylation gold kit (Zymo Research) or manually. This salt reduces the aromatic ring of an unmethylated cytosine, leading to an intermediate product that deaminates spontaneously. Subsequent addition of the NaOH base causes desulphonation of the ring, which is finally converted to a uracil ring (Fig. I-11). The reaction cannot occur on a methylated cytosine. The final effect is a DNA sequence change in a methylation-specific

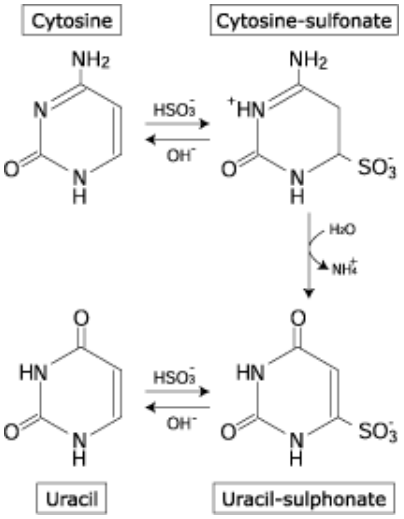


Figure I-11. Bisulphite modification reaction.

manner. DNA is depleted of all cytosines but those that are methylated. Uracils behave differently in base pairing, preferring an adenine to a guanine. Detection reactions are based either on hybridization to a probe that contains G (detecting methylation) or A (detecting unmethylation) in the position of interest, or on PCR amplification with primers specific for the modified DNA. The amplification product is sequenced, and will contain a C if the original DNA was methylated or a T if it was unmethylated.

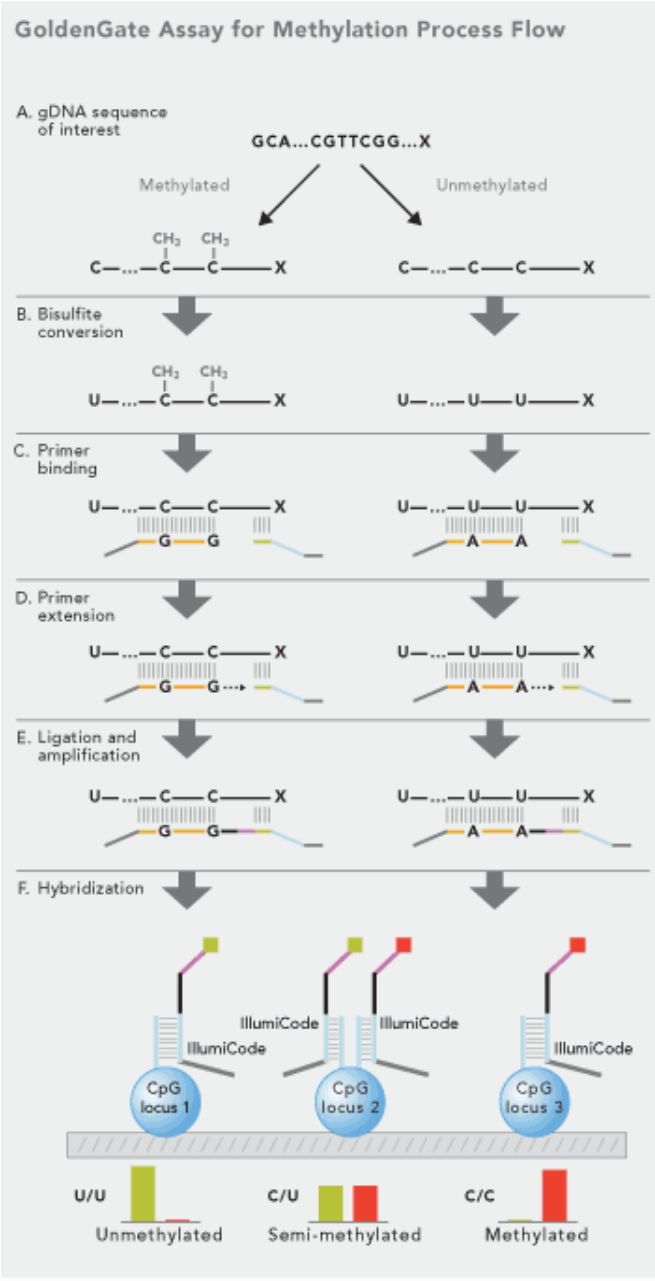
For the manual chemical reaction, 1 μg DNA (50 μL, final volume) was denatured with 5.7 μL of 3 M NaOH (37ºC, 10 min), then incubated with 33 μL of 16 mM hydroquinone and 530 μL of 4M sodium bisulphite ($NaHSO_3$; Sigma) pH 5.0 (50ºC, o/n). After purification by Wizard DNA-Clean Up kit (Promega) and elution in 50 μL H_2O, the reaction was terminated with 5.7 μL of 3 M NaOH (37ºC, 15-20 min). DNA was precipitated by adding 1 μL glycogen (10 mg/mL) solution, 17 μL of 7.5 M ammonium acetate and 450 μL cold absolute ethanol.

Bisulphite sequencing of multiple clones. DNA methylation was determined by PCR analysis. The region of interest was amplified from modified DNA with standard PCR, using primers designed by Methyl Primer Express Software (primer table). PCR products were analysed in 2% agarose gel electrophoresis and DNA was extracted using QIAquick Gel Extraction Kit and ligated into a pGEM-T easy plasmid. The plasmid was transformed in competent bacterial cells by the heat shock method and the transformation was plated onto LB/ampicillin/IPTG/X-Gal plates. A minimum of six white colonies of each sequence and sample were processed by miniprep (Montage Plasmid MiniprepHTS 96 Kit, Millipore) and plasmids were sequenced automatically to determine their degree of methylation. Sequences were aligned with the

Bioedit program and T (unmethylated) and C (methylated) were counted manually in potentially methylated CpG sites.

DNA methylation profiling using bead arrays

Goldengate arrays. The GoldenGate Methylation Cancer Panel I spans 1,505 CpG loci selected from 807 genes of which 28.6% contain one CpG site per gene, 57.3% contain two CpG sites, and 14.1% have three or more sites. Selected genes fall into various classes, including tumour suppressor genes, oncogenes, genes involved in DNA repair, cell cycle control, differentiation, apoptosis, X-linked, and imprinted genes. For each CpG site, four probes were designed: two allele-specific (ASO) and two locus-specific oligonucleotides (LSO). Each ASO–LSO pair corresponded to either the methylated or unmethylated state of the CpG site (Fig. I-12).



Bisulphite conversion of DNA samples was done using the EZ DNA methylation kit (Zymo Research). After bisulphite treatment, remaining assay steps were performed exactly using Illumina-supplied reagents and conditions. Once processed, image analysis and intensity data extraction were performed to obtain a value for the fluorescent signals from the methylated and unmethylated allele spot. Background intensity computed from a set of negative controls was subtracted from each analytical data point. The ratio of fluorescent signals was then calculated from the two alleles: a relative methylation value was quantified and standardized over a range from 0.0 to 1.0 (effectively 0% and 100% likelihood of gene promoter hypermethylation, respectively). In this study, all sequences with at least 70% likelihood of hypermethylation (hybridization signal $\geq$ 0.7) were considered hypermethylated for each specific sample, whereas sequences whose equivalent signal was below 30% (hybridization signal < 0.3) were considered hypomethylated.

To identify gene promoters that could be hypermethylated in a large number of samples of a specific group (hESC, NTT, and CCL), we selected all sequences whose hybridization signal was $\geq$ 0.7 in at least 25% of the samples for each group. In general, sequences were classified by a stepwise algorithm. First, according to the percentage of hESC hypermethylated in each specific probe set; sequences that were hypermethylated in $\geq$25% and <25% of samples were therefore considered hypermethylated and unhypermethylated, respectively.

Figure I-12. Goldengate assay workflow. (A) Only the top strand of the genomic DNA sequence of interest is shown. If other CpG sites are present in the vicinity of the target CpG site, it is assumed that they have the same methylation status as the site of interest. (B) Through a bisulphite conversion step, unmethylated cytosines are converted to uracils, while methylated cytosines remain unchanged. (C) For each CpG site, two pairs of probes are designed: an ASO (gold) and a LSO (green) probe pair for the methylated state of the CpG site and a corresponding ASO-LSO pair for the unmethylated state. Pooled oligos anneal to the target sequence. All loci are assayed simultaneously. (D) Extension occurs from the matched ASO toward the LSO. (E) Ligation (purple) of the extended ASO to the corresponding LSO created PCR templates. The ligated products are then amplified by PCR using fluorescent-labelled common primers, and hybridised to a bead array bearing the complementary address sequences. (F) The Illumina code (blue) contained within the LSO is hybridised to a complementary sequence on the bead array. Two fluorophores are then used to distinguish methylated from unmethylated loci. Locus one above shows an unmethylated site, locus two semi-methylated, and locus three, fully methylated. (from the Illumina website)

Sequences were then tested for hypermethylation in hCCL and classified according to the percentage (≥ 25% or < 25%) of hypermethylated samples in each probe set. Finally, the percentages of normal tissue types that were hypermethylated in each probe set were calculated, and sequences were classified as hypermethylated in all normal tissue types (100% of samples with signal ≥ 0.7), unmethylated in all normal tissue types (100% of samples with signal < 0.3) or unmethylated in some but not all samples (signal < 0.3 in at least one, but not all, samples). This algorithm allowed most sequences in the array to be assigned to one of the 12 groups described in Table 2. To determine the enrichment for a specific histone modification, all sequences were classified according to publicly available data on histone modification and Polycomb occupancy (Lee et al., 2006a; Mikkelsen et al., 2007; Zhao et al., 2007). A χ-square test was performed to identify significant differences in frequencies between the groups of sequences. Up to 27 tests were conducted so that Bonferroni-adjusted, two-tailed probabilities of < 0.0018 (0.05/27) were considered significant.

Infinium Methylation Assay. The HumanMethylation27 BeadChip interrogates 27,578 CpG sites per sample at single-nucleotide resolution, in the proximal promoter regions of transcription start sites of 14,475 consensus coding sequencing (CCDS) in the NCBI Database (Genome Build 36).

In addition, 254 assays cover 110 miRNA promoters. On average, two assays are present per CCDS gene and from 3 to 20 CpG sites for >200 cancer-related and imprinted genes. The assay is performed in a very similar way to that described above.

After bisulphite conversion (Fig. I-13), each sample was whole-genome amplified (WGA) and enzymatically fragmented. The product was later purified and processed following manufacturer's instructions. During hybridisation, the WGA-DNA molecules anneal to locus-specific DNA oligomers linked to individual bead types. The two bead types correspond to each CpG locus —one to the methylated (C) and the other to the unmethylated (T) state. Allele-specific primer annealing was followed by single-base extension using dinitrophenol- and biotin-labeled ddNTP. After extension, the array was fluorescently stained, scanned, and the intensities of the unmethylated and methylated bead types measured. To obtain DNA methylation values, analysis was performed using BeadStudio software (Illumina). DNA methylation beta values are continuous variables between 0 and 1, representing the ratio of the intensity of the methylated bead type to the combined locus intensity.

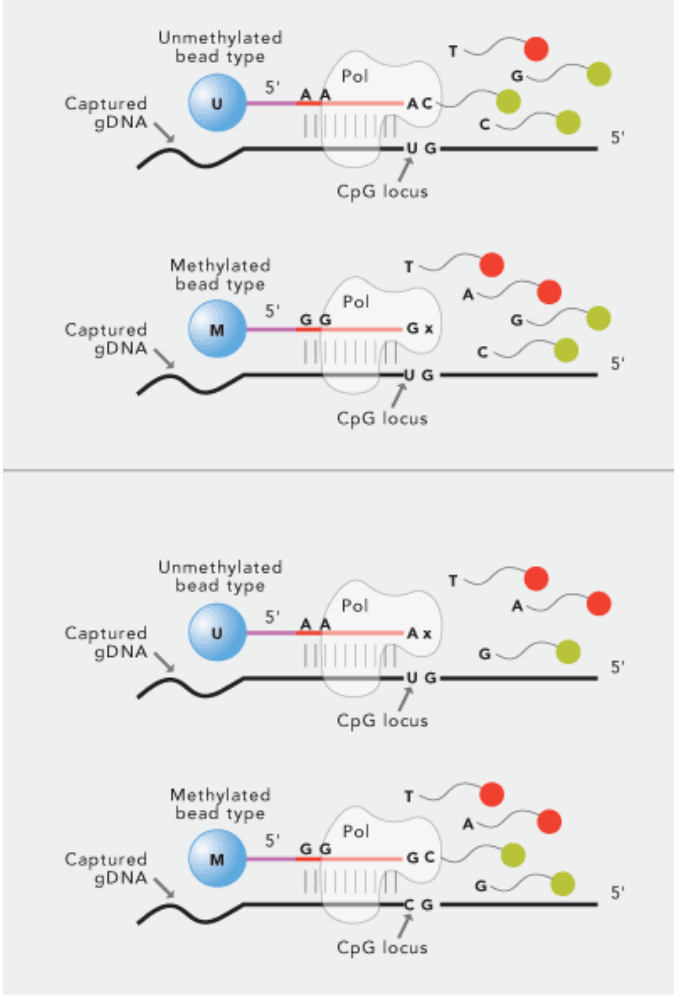


Figure I-13. Infinium assay scheme. The Infinium Methylation Assay uses two different bead types to detect CpG methylation. The U bead type matches the unmethylated CpG site; the M bead type matches the methylated site. In the top panels, the unmethylated CpG target site matches with the U probe, enabling single-base extension and detection. It has a single-base mismatch to the M probe, which inhibits extension. If the CpG locus of interest is methylated (bottom panels), the reverse occurs. (From the Illumina website)

mRNA quantification

RNA extraction and real-time RT-PCR analysis. Total RNA was isolated with TRIzol Reagent (Invitrogen). Cell pellets were resuspended in 1 ml TRIzol and, after addition of 200 µl chloroform and phase extraction of RNA, aqueous phase was isolated. RNA was precipitated with isopropanol and finally resuspended in H_2O, after which 2 µg of total RNA were treated with Turbo RNAse-free DNAse (Ambion; 37ºC, 10 min) and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit according to manufacturer's protocols. Approximately 1/100 of the RT reaction (corresponding to 20 ng of original RNA) was normally tested in triplicate in real-time PCR. Quantitative real-time RT-PCR was performed in

some cases (see primer table) using 2X TaqMan PCR Master Mix and TaqMan Gene Expression Assays, pre-designed validated assays containing a primer pair and a TaqMan probe for the transcript of interest (Applied Biosystems). In other cases, we performed q-RT-PCR with SYBR Green Universal PCR Master Mix, associated with primers we designed with Primer Express software, generally extracting from the Ensembl database the most abundant transcript sequence for each gene and choosing an amplicon overlapping an exon-exon junction, to avoid amplification in possible genomic DNA contamination (see primer table for primers and assays). Some samples were assayed by the low-density TaqMan Human and Mouse Stem Cell Pluripotency Array and the ABI PRISM 7900 sequence-detection system (Applied Biosystems). These low-density cards are pre-loaded with 96 validated Taqman assays. The Pluripotency Arrays contain 6 control genes, 42 genes related to pluripotency and 50 differentiation genes. These cards are loaded with diluted cDNA-2 X Taqman Master Mix with a microfluidic system that allows automatic sample distribution in all 384 wells by centrifugation.

mRNA stability assay. Cells were treated with 5 μg/ml actinomycin D (Sigma) at the times indicated and immediately resuspended in TRIzol reagent for RNA extraction.

mRNA immunoprecipitation. Pellets were homogenized in lysis buffer [100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.0, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), RNase OUT (100 U/ml), and complete protease inhibitor cocktail (1:1000)], centrifuged and protein concentration was measured in the supernatant using the Bradford assay. Fresh whole-cell lysate was first precleared with 15 μg IgG1 mouse control (BD Pharmingen), and 25 μl of beads (protein A-Sepharose, Sigma; 4ºC, 30 min). Beads were incubated overnight with 30 mg of IgG1 or anti-HuR antibody (Santa Cruz Biotechnologies), washed twice using NT2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% Nonidet P-40) and incubated with the cleared lysate (4°C, 1 h). The pellet was washed four times in NT2 buffer, then resuspended in 100 μl NT2 buffer containing 20 U RNase-free DNase I (Invitrogen; 37°C, 15 min), washed with NT2 buffer, and eluted in 100 μl NT2 buffer containing 0.1% SDS and 0.5 mg/ml proteinase K (Roche; 55°C, 15 min) and collected. RNA was extracted with acid phenol-chloroform and precipitated overnight in the presence of 5 μl glycoblue (Ambion), 25 μl sodium acetate pH 5.2, and 625 μl 100% ethanol.

Protein detection and analysis

Western blot analysis. For protein analysis, cells were lysed with RIPA (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, complete protease inhibitor cocktail) or directly in Laemmli buffer (4X = 4% SDS, 20% glycerol, 10% BME, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) and analysed by Western blot. SDS polyacrylamide gel electrophoresis (PAGE) was performed using gels with a polyacrylamide concentration ranging from 8% to 12% (up to 15% for histone H4), based on the size of the protein to be detected. Protein were transferred to PVDF membranes (Immobilon, Millipore) by semi-dry transfer (Biorad or Invitrogen) and, after blocking with 5% fat-free milk (Difco), were incubated with primary antibodies to α-tubulin (Sigma-Aldrich), SirT1, phospho-Ser27 SirT1, p53, acetyl-lysine-382 of p53, OCT4 and SOX2 (all from Cell Signaling Technologies), acetyl-K16 of histone H4 (Active Motif), HuR and V5 (Invitrogen), methyl-HuR (Li et al., 2002), CARM1 (Biovision), NANOG (R&D Biosystems) and E-cadherin (Becton Dickinson). Secondary antibodies used were HRP conjugated-anti-mouse IgG, -rabbit IgG and -goat IgG (Amersham). Membranes were later developed with ECL or ECLplus reagents (Amersham) and exposed to autoradiography films (Agfa or Amersham). To purify nuclear and cytoplasmic fractions, cytosolic proteins were first extracted by incubating the cell pellet in RSB buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP40, complete protease inhibitor cocktail) for 15 min on ice with gentle vortexing, then separated from the nuclear pellet by centrifugation.

Immunofluorescence. EB were suspended in 50 μL HF (Hank's Balanced Salt Solution [Invitrogen], 2% FBS), fixed and permeabilised with 100 μL Intraprep Reagent 1 (Beckman Coulter; 37°C, o/n). After washing five times each for 1 h with HF 4% BSA, EB were resuspended in 100 μL Intraprep Reagent 2 and 5 μL 4% BSA with the appropriate primary antibody (37°C, o/n). They were then washed 5 times, resuspended in 100 μL HF with 5 μL 4% BSA with the secondary antibody. For imaging, hEB were dispensed onto a microscope slide, mounted in a drop of Mowiol (10% (w/v) Mowiol (Sigma), 25% glycerol (w/v) and 0.1 M Tris-HCl, pH 6.8, 2.5% DABCO (v/v)), then covered with a coverslip. hEB were then imaged by laser-scanning confocal microscopy. Adherent cells were fixed in 3.7% formaldehyde (10 min, 37°C), washed 3x in PBS,

permeabilised with 100% methanol (3 min, RT), washed 3x in PBS, and blocked (1 h) in PBS with 5% BSA. Primary antibody was added in 2% BSA in PBS and incubated 2 h. Cells were then washed 3x in PBS and secondary antibody was added in PBS with 10% FBS. Cells were then incubated (2 h, RT), washed 3x in PBS, and imaged in Mowiol. The first antibody was anti-SirT1, followed by Alexa448 goat anti-rabbit IgG (Molecular Probes).

HuR phosphorylation assay

Phosphorylated-HuR (Ser) was immunoprecipitated using an agarose-conjugated anti-phosphoserine antibody (Sigma). Samples were homogenised in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, complete protease inhibitor cocktail, complete phosphatase inhibitor cocktail, 1 mM orthovanadate, 1 mM NaF), and 100 µg of total protein were incubated overnight with 25 µl agarose-conjugated anti-phosphoserine in a total volume of 500 µl. Immunoprecipitated HuR was washed three times with lysis buffer and the pellet dissolved in SDS sample buffer, boiled (95°C, 5 min), and after centrifugation the supernatants was loaded onto polyacrylamide gels. Finally, HuR phosphorylation (immunoprecipitation) was detected by Western blot using an anti-total HuR monoclonal antibody (Santa Cruz Biotechnology).

High-performance liquid chromatography (HPLC). Histones were prepared by acid extraction of protein from the cell pellet by two rounds of lysis in 0.25 M HCl (o/n, 4ºC) followed by centrifugation and acetone precipitation. Once total basic protein was obtained, histone H4 was purified by reverse-phase HPLC on a Jupiter C18 column (Phenomenex) with an acetonitrile gradient (20−60%) in 0.3% trifluoroacetic acid, using a HPLC gradient system (Beckman-Coulter).

Top-down mass spectrometry. High-resolution mass measurements for exact mass determination were carried out using an APEX Qe Fourier transform mass spectrometer (Bruker Daltonics) equipped with a 9.4 tesla superconducting refrigerated cryomagnet and external electrospray ion source (Dual source). The spectra were externally calibrated with Arginine cluster in positive ion mode in the mass range 350–1400 m/z. The stock solutions of histone samples were diluted 1:20 in 50% methanol + 0.2% formic acid and introduced into the electrospray ion source using a syringe pump with a flow of 2 µL/min. Spectra were acquired over a mass range of 200–3000 m/z using 1M data points. After sine apodization, spectra were processed with DataAnalysis 3.4 (Bruker Daltonik GmbH) using SNAP2 for quantification.

Transfection, heterologous protein expression and knockdown

RNA interference. siRNA transfection mix was prepared by separately adding 5 µl 20 mM siRNA (small interference RNA; see primer table) to 75 µl OptiMEM (Gibco) and 1.5 µl Lipofectamine RNAiMAX (Invitrogen) to another 75 µl OptiMEM aliquot. After 5 min, diluted siRNA was mixed with Lipofectamine, mixed and incubated 1 h to allow complex formation. For siRNA transfection in hESC, a single cell suspension was generated. hESC flasks were washed with PBS and treated with Accutase (Millipore) diluted 1:10 in PBS (3 ml per T75 flask). After several minutes incubation at RT, with tapping and constant checking for colony disgregation, incubation was terminated when colonies were nearly completely disgregated, but neither the feeders nor the differentiated edges. The cell suspension was collected and attached cells were washed again with PBS. Remaining clumps in the suspension were disgregated by pipetting and cells were passed through a cell strainer (40 µm) to eliminate clumps and feeders. After centrifugation, cells were resuspended in mTeSR (Stem Cell Technologies) at 37ºC. Finally, cells were counted and brought to a final concentration of 2.5 x 10^5 cells/ml, seeded at 0.5 ml/well in a Matrigel (BD Biosciences)-precoated 12-well plate, and 150 µl siRNA mix was added immediately, drop by drop, and further mixed by gentle tapping. After 24 h, mTESR medium was substituted daily for three or four days.

For EB formation, the same transfection protocol was scaled up to P-100 plates. At day 2 post-transfection, cells were scraped and aggregates resuspended in EB formation medium as above. These aggregates were collected at days 3 and 8 post-Eb induction.

Plasmid construction and transfection. The full-length HuR cDNA was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH. The HuR (WT)-V5 was constructed by PCR amplification using the 5´oligonucleotide containing the V5 tag sequence and subcloned into pCDNA 3.3 TOPO vector (Invitrogen). The HuR (R217K)-V5 and HuR (R217A)-V5 mutants were constructed using the QuickChange kit for site-directed mutagenesis (Stratagene) with two complementary oligonucleotides and

the pCDNA-HuR (WT)-V5 plasmid as template. Primers are shown in the primer table. All plasmids were cloned by competent bacteria transformation, as described in the bisulphite sequencing section, and final plasmids for hESC transfection were obtained with the Genopure Plasmid Midi kit (Roche). For plasmid transfection, Shef-1 hESC were prepared as a single cell suspension as described in the siRNA interference section. Cells (2 x 10^6) were pelleted for each reaction and transfected with 4 µg plasmid using the Human Stem Cell Nucleofector kit 2 (Amaxa; Lonza) with the transfection programme A-23 and following supplier's instructions. Nucleofected hESC were seeded in 6-well Matrigel-coated plates and maintained for 3 days in mTESR1, with daily medium changes.

Chromatin analysis

Chromatin immunoprecipitation. Samples for ChIP assay were collected by fixing with 1% formaldehyde final concentration (Sigma) for 15 min, then arresting the reaction with glycine (0.125 M final concentration), washing twice with PBS supplemented with complete protease inhibitor cocktail, and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1; 200 µl/10^6 cells). After lysis (10 min, on ice), lysates were sonicated in a Branson Sonifier 450 bath (Diagenode; 20 min at maximal intensity, 30 sec on/off cycles). Soluble sonicated chromatin was then separated by centrifugation from cell debris and approximate concentration measured by Nanodrop (260 nm). Chromatin (5-10 µg) were then diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton-X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and immunoprecipitated (o/n, 4ºC) with 5-10 µg antibody [anti-acK9H3 (Millipore), -AcK16H4, -SirT1 and anti-H3 (Abcam)]. Immune complexes were collected with Salmon Sperm DNA/Protein A Agarose-50% Slurry (1 h, 80 µl/sample), washed sequentially with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffers, then eluted with 500 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$), decrosslinked (0.2 M NaCl, o/n, 65ºC), treated with Proteinase K (0.01 M EDTA, 0.04 M Tris-HCl, pH 6.5, and 20 mg Proteinase K; 1 h, 45ºC), phenol-chloroform-extracted and precipitated.

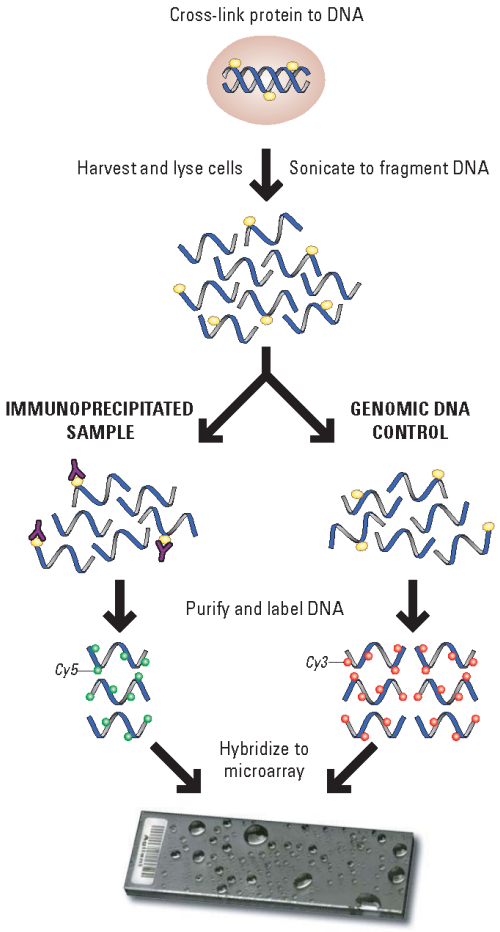


Figure I-14. ChIP on chip flowchart. From Agilent website.

ChIP-on-chip. For the ChIP-on-chip assay, we used the Agilent Human Promoter ChIP-on-chip Microarray Set. It contains, in two 244K chips, probes that cover from -5.5 kb upstream to +2.5 kb downstream of the transcription start sites for ~17,000 of the best-defined human transcripts represented by RefSeq, sourced from UCSC hg18 (NCBI Build 36.1, March 2006). SirT1-immunoprecitated DNA (4 µg) fragments and the corresponding unbound fractions were Cy3- and Cy5-labelled and hybridised to the array slides at the CNIO Genomic facility, following manufacturer's instructions (Fig. I-14). Results were analysed using the Agilent DNA Analytics program (Lee et al., 2006a). In Table S13, we show the probes considered positive after applying feature extraction protocol, setting the following criteria: (i) blank subtraction normalization, maximum distance (in bp) for two probes to be considered neighbours = 500; (ii) a probe is considered bound if P[X bar] $<10^{-3}$ and either central probe of the peak has P[X] $< 10^{-3}$ and at least two neighbouring probes have P[X] $<10^{-2}$ or at least one of the neighbours has P[X] $< 5{\cdot}10^{-3}$. Gene ontology was examined with DAVID (Dennis et al., 2003) (Huang et al., 2009), a web-based tool. Results are shown in Table S14 as a GO chart and in Table S15 as GO clustering.

Quantitative ChIP. q-PCR reactions on immunoprecipitated DNA were performed with human-specific primers, designed with the Primer Express software to amplify the genomic region surrounding the SirT1-binding probe for each gene, 2X SYBR Green PCR Master Mix using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The enrichment factor refers to the copy number of a gene of interest in the bound fraction after ChIP with the appropriate antibody, divided by the corresponding copy number of that gene in the unbound form for that antibody. For SirT1, immunoprecipitation data are expressed as the percent enrichment of the SirT1 immunoprecipitated sample relative to the negative control (no antibody). For histone mark immunoprecipitation, where two samples (ES and EB) were compared, data were further normalised for the total histone H3 signal.

RESULTS

RESULTS

PART I: DNA methylation in hESC differentiation and developmental gene activation

DNA methylation study by amplification of intermethylated sites in hESC differentiation

We first approached the study of DNA methylation changes during human embryonic stem cell differentiation *in vitro* through the AIMS technique. We used DNA from hESC Shef-1 and I3 and their differentiated counterparts (Shef-1 embryoid bodies [EB] and I3 neural precursors [NP]). AIMS is based on the differential enzymatic digestion of genomic DNA with methylation-sensitive and -insensitive isoschizomers, followed by restrained PCR amplification of methylated sequences. Further processing allows visualization of amplified DNA on a sequencing gel and identification of bands of interest by cloning and sequencing. We observed several bands whose unequal appearance indicated distinct methylation status in the undifferentiated-differentiated pair (Fig. 1).

By cloning and sequencing these bands, we identified 23 methylated sites, eight of which appeared to be differentially methylated in at least one cell line (Table 1), five of them were identified as repetitive sequences, in which methylation is concentrated mainly in the genome, and two were mouse sequences, probably due to DNA contamination from MEF, used as feeders in the hESC culture. This method was not suitable for genomic analysis in our system, and the CpG identified were too often found in repetitive sequences or in intergenic regions for which a functional correlation with gene expression was improbable. We therefore turned to other, more global methods using a promoter-focused approach.

Promoter methylation profiling in hESC, normal tissues and cancer using Goldengate array

We scaled up the analysis to determine which promoters are regulated by DNA methylation during hESC differentiation, using Goldengate Methylation Arrays, Cancer Panel 1 (Illumina). These commercial arrays contain 1,505 sequences (from 807 genes) chosen on the basis of their importance to cell behaviour

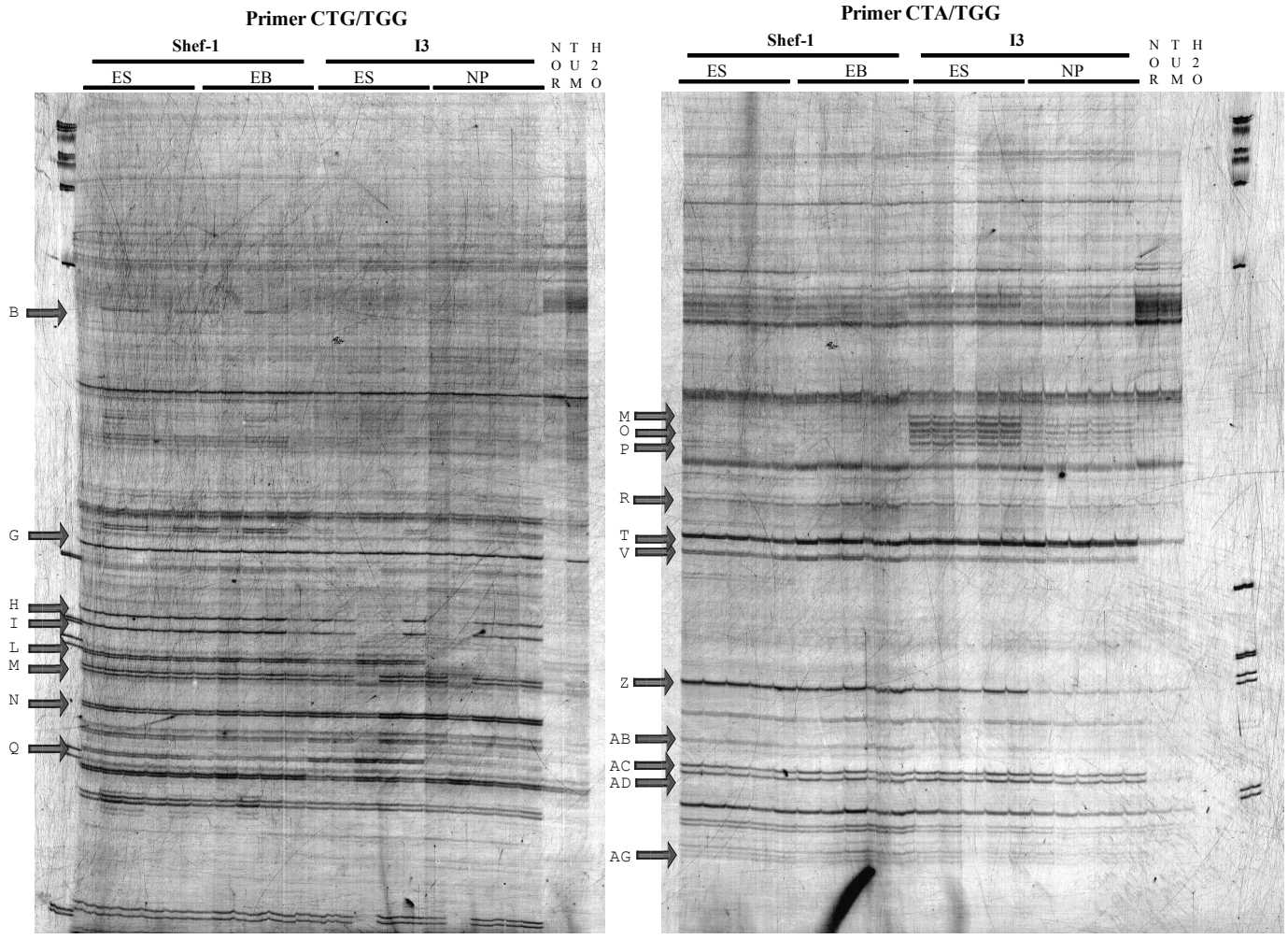


Figure 1. AIMS analysis of genomic DNA from undifferentiated embryonic stem cells (ES) and differentiated embryonic bodies (EB) or neural precursor (NP) samples of Shef-1 and I3 cell lines. Control DNA samples of normal lymphocyte (NOR) and tumour tissue (TUM) are shown for each gel (right). Sequenced bands cited in Table 1 are indicated (arrow); letters refer to nomenclature in Table 1, first column.

Name	Chromosome	Adjacent gene 5' or containing sequence	Adjacent gene 3'	Differential methylation
AIMS_CTA_AB	9	3115: ZMYND19	12137: ARRDC1	
AIMS_CTA_AC	rep			
AIMS_CTA_AD	11	126: FAM99A	30386: KRTAP5-6	
AIMS_CTA_AG	Mm6			
AIMS_CTA_M	rep			Yes
AIMS_CTA_O	rep			Yes
AIMS_CTA_P	rep			Yes
AIMS_CTA_R1	5	127449: FLJ44606	144559: MEGF10	
AIMS_CTA_R2	6	JARID2		
AIMS_CTA_T	10	RSU1		
AIMS_CTA_V	Mm11			
AIMS_CTA_Z	3	BOC		Yes
AIMS_CTG_B	2	HDAC4		Yes
AIMS_CTG_G	11	123528: MYEOV	222452: CCND1	
AIMS_CTG_H	8	28526: HOOK3	8708: FNTA	
AIMS_CTG_H3	17	6051: CRLF3	1408: ATAD5	
AIMS_CTG_I	10	546166: GLRX3	367378: TCERG1L	
AIMS_CTG_L	11	123: FAM99A	30386: KRTAP5-6	Yes
AIMS_CTG_M	X	58798: KLF8	220390: UBQLN2	
AIMS_CTG_N	2	147984: SERPINE2	250328: FAM124B	
AIMS_CTG_N2	19	GNA11		
AIMS_CTG_Q	rep			Yes
AIMS_CTG_Q2	16	ATP2A1		Yes

Table 1. Identification of sequenced bands in AIMS gels (Fig. 1). Chromosomal location and distance from adjacent 5' and 3' genes are shown; where no distance is indicated, the sequence falls within the gene cited.

in cancer and differentiation, and include genes reported to be differentially methylated, as well as tumour suppressor genes, oncogenes and genes coding for factors involved in cell cycle check point. The main advantages of this assay are that it permitted simple, rapid analysis of a considerable number of samples, rendering statistical analysis possible, and the promoter-directed design. In this platform, we compared DNA methylation status in eight independently isolated hESC lines and 21 normal human primary tissues (NPT) corresponding to six normal tissue types (NTT). As this array is cancer-directed, we included 21 human cancer cell lines (CCL) to determine whether the hESC promoter methylation profile resembles that observed in cancer cells (Bibikova et al., 2006). Given the variety of sample origins, we selected autosomal genes (766) from the arrays to exclude DNA methylation-dependent X-inactivated genes (Fig. 2).

Unsupervised clustering of the samples using exclusively the methylation signals of the autosomal genes contained in the arrays (1,421 sequences) enabled correct classification of each sample within its corresponding group (hESC, NPT, or CCL) (Fig. 2), confirming that each sample group has a specific DNA methylation signature (Bibikova et al., 2006). An overall appraisal of the array results confirmed that some genes are repressed by promoter hypermethylation during hESC differentiation, but this phenomenon is uncommon among the genes represented in this array. On the contrary, we determined that a considerable proportion (12.5%, 177/1421) of gene probes undergo demethylation during ES differentiation to at least one normal tissue. Moreover, a large proportion of these genes are hypermethylated in cancer. We thus classified the gene probes in the array relative to their methylation status in the three sample types (hESC, CCL, and NPT), in a way that we consider helpful for understanding both the process of promoter demethylation during hESC differentiation and the possible relationship between hESC and cancer promoter hypermethylation (Fig. 3A; Tables 2 and S2). We found that 65.31% (928/1,421) of the sequences were not frequently hypermethylated in hESC (array signal ≤ 0.7 in $\geq 25\%$ (2/8) of samples) and that half (464/928) were frequently hypermethylated in CCL (array signal > 0.7 in $\geq 25\%$ (6/21) of samples). The vast majority of these sequences (99.78%, 463/464) were unmethylated in at least one NTT

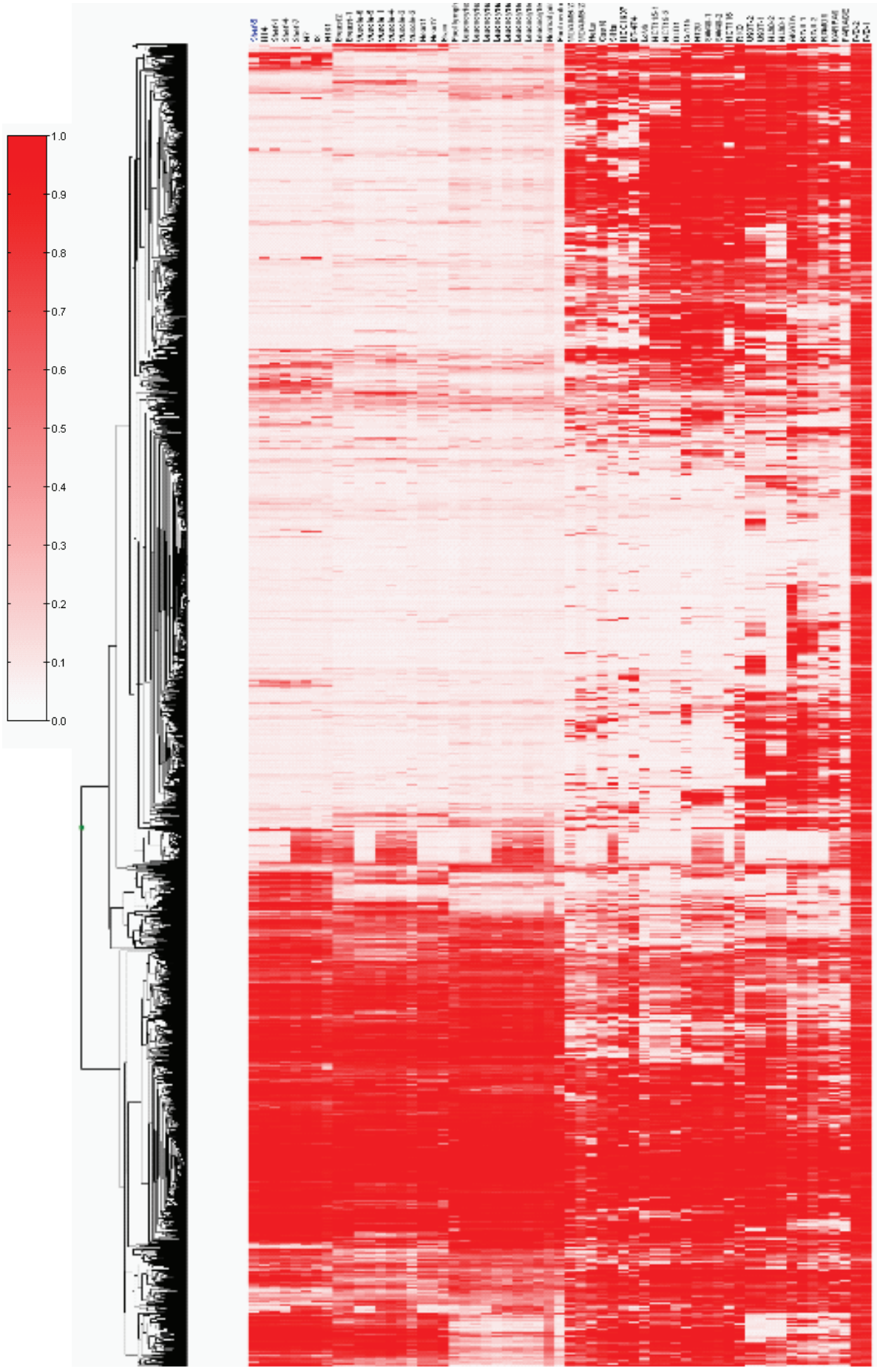


Figure 2. Unsupervised cluster analysis of hESC, hCCL, and NPT based on correlation of methylation profiles of 1,421 sequences. Methylation levels vary from fully methylated (red) to fully unmethylated (white) sequences. The last two rows correspond to in vitro-methylated DNA (IVD), used as a positive control for methylation.

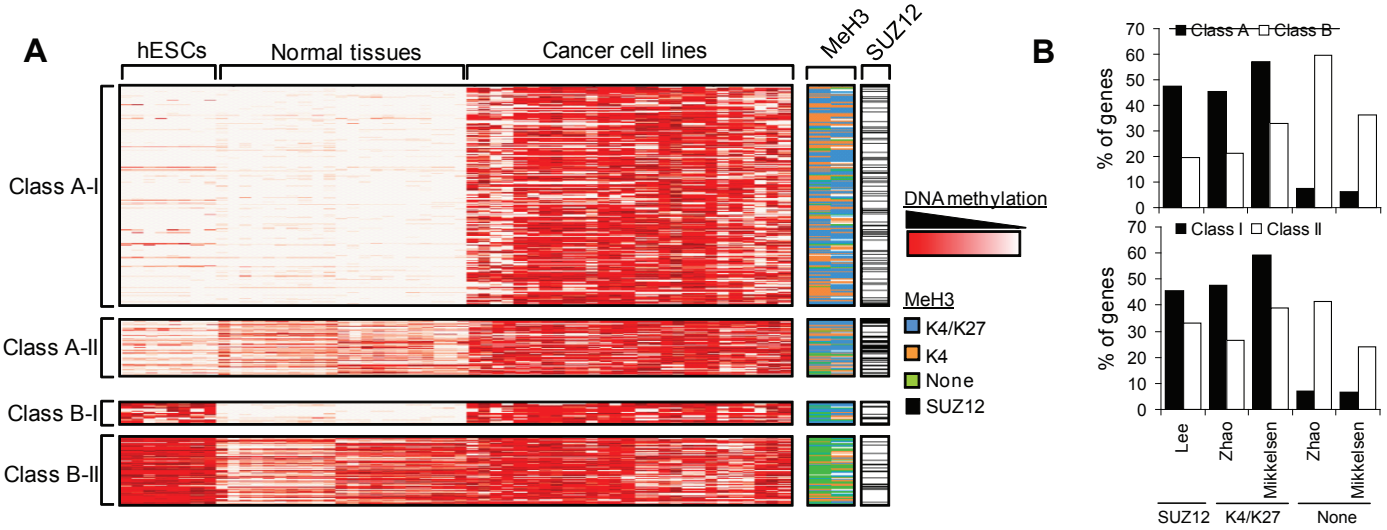


Figure 3. DNA methylation profiling in hESC, NPT and CCL. (A) Methylation profiles of Class A-I (350), A-II (94), B-I (20), and B-II (107) genes in hESC (8), NPT (21), and CCL (21) samples obtained in Illumina arrays. The methylation levels varied from fully methylated (red) to fully unmethylated (white). The right-hand columns show the methylation status of histone H3 and Polycomb occupancy of the same genes obtained from previously published data (Lee et al., 2006a; Mikkelsen et al., 2007; Zhao et al., 2007). Blue, methylation at K4 and K27; orange, methylation at K4 alone; green, no methylation at K4 or K27; black, presence of the Polycomb protein SUZ12. (B) Enrichment of Polycomb protein SUZ12, the bivalent chromatin signature (K4/K27) or the absence of both marks (none) in Class A and B genes (top), and Class I and II genes (bottom).

analyzed (array signal < 0.3 in ≥ 1/6 NTT). This finding is consistent with the view that genes aberrantly hypermethylated in cancer (i.e., not hypermethylated in normal tissues) are not hypermethylated in hESC (Ohm et al., 2007). We called this group of genes classical Class A cancer hypermethylated genes (Tables 2 and S1).

We found that 34.69% (493/1,421) of the sequences were frequently hypermethylated in hESC; most of these (79.72%, 393/493) were also frequently hypermethylated in CCL. Again, many (32.32%, 127/393) were unmethylated in at least one of the NTT analysed (Fig. 2; Tables 3 and S1). In contrast to Class A cancer hypermethylated genes, those of this group were also frequently hypermethylated in hESC; we thus propose that they form a distinct category, which we have termed Class B cancer hypermethylated genes. Of the 697 sequences frequently hypermethylated in cancer and unmethylated in at least one of the NTT analyzed, 444 (66.70%) and 127 (18.22 %) were classified as Class A and Class B genes, respectively (Fig. 3A; Tables 2 and S2). This indicates, contrary to predictions, that a substantial proportion (~20%) of cancer methylated genes is also frequently hypermethylated in hESC.

Not all genes frequently hypermethylated in CCL were completely unmethylated in all NPT analysed (Fig. 3; Tables 2 and S1). Hypermethylation frequency in NPT is only of moderate importance for Class A cancer methylated genes, as most of them (78.83%; 350/444 sequences) are unmethylated in NPT. On the other hand, only 20 sequences corresponding to Class B genes were unmethylated in all NTT analysed (Table 2).

When a gene is methylated in some but not all normal tissues, this methylation is probably involved in the specification of a tissue type during development (Zhao et al., 2007). If the gene is not hypermethylated in hESC, tissue type-dependent selective methylation must occur; in contrast, if the gene is frequently hypermethylated in hESC, it is probably selectively demethylated upon differentiation, as an epigenetic mechanism that facilitates tissue specification. Conversely, when a gene is unmethylated in all normal differentiated cells and hypermethylated in stem cells, the loss of promoter methylation that necessarily occurs during differentiation is more likely to be involved in early differentiation processes than in tissue specification (Zhao et al., 2007). We therefore defined two subcategories for both Class A and B cancer methylated genes: subcategory I for genes always unmethylated in normal tissues, and subcategory II for genes sometimes methylated in normal tissues (Fig. 3; Tables 2 and S2). The percentage of Class A-II and B-II genes is quite similar (7.53% and 6.61%; Table 2). The percentage of genes in Class A-I (24.63%) is nonetheless much higher than that in Class B-I (1.41%). Genes in these four categories (A-I, A-II, B-I, B-II) represent 58.2% of all sequences in the methylation arrays. Considering the methylation status of the three groups (hESC, NPT and CCL), we clustered most remaining genes in the array into eight additional categories (Table 2). These included, for example, two categories of genes that we define as being constitutively methylated (methylated in hESC, CCL and all NTT; 11.19%) or constitutively unmethylated (unmethylated

	Methylation in hESC	Methylation in CCL	Methylation in NTT	Proposed biological role	Name	Groups in Table S2
1421 sequences	Hypermethylated in hESC > 0.7 in > 2/8 samples 493 sequences (34.69%)	Hypermethylated in hCCL > 0.7 in > 6/21 samples 393 sequences (27.66%)	159 sequences (11.19%) hypermethylated in all NTT (> 0.7 in 6/6 samples)	Genes constitutively hypermethylated	-	G1
			20 sequences (1.41%) unmethylated in all NTT (< 0.3 in 6/6 samples)	Genes that become demethylated early in hESC differentiation, or aberrantly hypermethylated during hESC *in vitro* culture. Their hypermethylation might provide advantages to the cancer cells.	Class B-I	G2
			107 sequences (7.53%) sometimes unmethylated (≤ 0.3 signal in ≥ 1/6 and ≤ 5/6 samples)	Genes whose demethylation during hESC differentiation might be important for lineage specification. Their hypermethylation might provide advantages to the cancer cells.	Class B-II	G3
		Not hypermethylated in hCCL Not > 0.7 in > 6/21 samples 100 sequences (7.04%)	16 sequences (1.13%) hypermethylated in all NTT (> 0.7 in 6/6 samples)	Genes that are frequently demethylated in cancer	-	G4
			11 sequences (0.77%) unmethylated in all NTT (< 0.3 in 6/6 samples)	Genes that are demethylated early during hESC differentiation. Their hypermethylation might not provide advantages to the cancer cells.	-	G5
			39 sequences (5.07%) sometimes unmethylated (≤ 0.3 signal in ≥ 1/6 and ≤ 5/6 samples)	Genes whose demethylation during hESC differentiation might be important for lineage specification. Their hypermethylation might not provide advantages to the cancer cells.	-	G6
	Not hypermethylated in hESC Not > 0.7 in > 2/8 samples 928 sequences (65.31%)	Hypermethylated in hCCL > 0.7 in ≥ 6/21 samples 464 sequences (32.65%)	1 sequence (0.07%) hypermethylated in all NTT (> 0.7 in 6/6 samples)	Genes hypermethylated early in hESC differentiation. Their hypermethylation should not provide advantages to the cancer cells.	-	G7
			350 sequences (24.63%) unmethylated in all NTT (< 0.3 in 6/6 samples)	Genes constitutively unmethylated during normal development. Their aberrant hypermethylation provides advantages to the cancer cells.	Class A-I	G8
			94 sequences (6.61%) sometimes unmethylated (0.3 signal in ≥ 1/6 and ≤ 5/6 samples)	Genes whose hypermethylation during hESC differentiation might be important for lineage specification. Their aberrant hypermethylation provides advantages to the cancer cells.	Class A-II	G9
		Not hypermethylated in hCCL Not > 0.7 in ≥ 6/21 samples 464 sequences (32.65%)	1 sequence (0.07%) hypermethylated in all NTT (> 0.7 in 6/6 samples)	Genes hypermethylated early in hESC differentiation. These genes could be aberrantly hypomethylated in cancer.	-	G10
			404 sequences (28.43%) unmethylated in all NTT (< 0.3 in 6/6 samples)	Genes constitutively hypomethylated	-	G11
			52 sequences (3.66%) sometimes unmethylated (≤ 0.3 signal in ≥ 1/6 and ≤ 5/6 samples)	Genes whose hypermethylation during hESC differentiation might be important for lineage specification. These genes could be aberrantly hypomethylated in cancer.	-	G12

Table 2. Classification of genes according to their promoter methylation status in hESC, NPT and CCL, and proposed biological role for each group

in hESC, CCL, and all NTT; 28.43%). We suggest that DNA methylation is not important for regulation of the genes in these categories. The classification of genes according to their methylation status in hESC, CCL and NTT, and the interpretation of the biological role of DNA methylation in the genes in each group is summarized in Table 2. Table S2 lists the genes in each group.

It is important to note here that all the previously described percentages refer to the 807 genes included in methylation arrays, whereas the overall percentage of genes in each group might be different if the entire genome were considered. The classification threshold that we employed to identify genes frequently hypermethylated in hESCs (more than 70% of promoter CpG methylation in more than 25% of samples analyzed) is that which is commonly used to define a gene as being frequently hypermethylated in cancer (Fraga et al., 2008). To assess whether our observations hold true for astringent classification thresholds we re-examined our data in search of: i) sequences hypermethylated in most of the hESCs analyzed (array signal > 0.7 in ≥ 75% (6/8) of the hESCs) and, ii) sequences "fully methylated" in some of the hESCs analyzed (array signal > 0.8) in ≥ 25% (2/8) of the hESCs (Table S3). We found that 5 B-I and 84 B-II sequences fitted the first criterion, and 13 B-I and 86 B-II sequences fitted the second (Table S3), which indicates that our conclusions remain valid even with these stricter classification thresholds.

Prolonged *in vitro* culture of hESC is associated with instability of DNA methylation (Allegrucci et al., 2007; Maitra et al., 2005). To assess whether promoter hypermethylation of Class B genes is associated with the *in vitro* culture process, we compared our data with those of Bibikova *et al*. (Bibikova et al., 2006). These authors used the Goldengate methylation platform to compare the methylation status of the 1505 CpG sites contained in the arrays in 10 hESC lines at low and high passage numbers. Although they found methylation changes with passage number, these changes were small compared to the differences among cell types. They found that five genes (ASCL2, GALR1, MEST, NPY, and SLC5A8) were consistently hypermethylated with passage number (increase in methylation level > 0.34 in at least two cell lines (20%)). Three of those genes (ASCL2, NPY, and SLC5A8) are members of Class B-I, but none was a Class B-II gene. This comparison suggests that prolonged *in vitro* culture was only responsible for promoter hypermethylation of a small fraction of Class B genes (3/97, 3%), and that the effect was greater in Class B-I genes.

It was recently proposed that developmental genes are silenced in ESC by a Polycomb-dependent bivalent histone-based chromatin mark (Bernstein et al., 2006; Lee et al., 2006a), which is thought to predispose to aberrant DNA promoter hypermethylation of TSG in cancer (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007). As we found that a subset of cancer methylated genes can also be methylated in hESC, we investigated the relationship between promoter hypermethylation and the Polycomb-dependent histone modification pattern in hESC. We compared our methylation data for Class A-I, A-II, B-I, and B-II genes with the previously reported histone modification profile and Polycomb occupancy of the same genes in embryonic stem cells (Lee et al., 2006a; Mikkelsen et al., 2007; Zhao et al., 2007) (Fig. 2; Table S4). Consistent with an earlier report (Widschwendter et al., 2007), we found that ~35% of the sequences frequently hypermethylated in cancer and unmethylated in at least one of the NTT analysed contained chromatin-repressive marks at their promoters (228-277/697 harboured meK27, and 236/697 contained SUZ12). Comparison of our methylation data with those of Mikkelsen *et al*. (Mikkelsen et al., 2007), we observed that the great majority (96.4%) of genes harbouring meK27 also contained meK4, and that only ~30% of the genes frequently hypermethylated in cancer presented the bivalent chromatin domain (trimethyl-K4/ trimethyl-K27) in hESC (Table S4).

When we compared chromatin patterns and Polycomb occupancy in the Class A-I, A-II, B-I, and B-II genes, we found that each group had a specific chromatin signature ($p < 0.00001$). Class A genes were more enriched in Polycomb and bivalent marks (47.5% and 45.5-57.3% of genes, respectively) than Class B genes (19.7% and 21.4-32.7%, respectively) ($p < 0.00001$) (Fig. 3B; Table S5). Enrichment of the bivalent mark in Class A genes is primarily due to the low levels of this chromatin signature in Class B-II genes (Table S4). Levels of meK4/meK27 in Class B-I genes were similar those in Class A genes ($p < 0.00001$; Table S5). Class II genes were much less frequently occupied by Polycomb proteins and had fewer bivalent marks (33.3% and 26.5-38.8%s, respectively; $p < 0.00001$) than did Class I genes (45.7% and 47.6-59.3%, respectively; Fig. 3B; Table S5). Lower levels of the bivalent mark in Class II genes were due primarily to the low levels of this chromatin signature in Class B-II genes (Table S5); Class A-II genes had similar meK4/meK27 levels to those of Class I genes ($p < 0.00001$; Table S5). We thus conclude that class B-II gene promoters are depleted of both histone marks and are likely to be kept silenced in hESC by promoter hypermethylation

rather than a Polycomb-dependent, histone modification-based mechanism.

TSG repressed by promoter hypermethylation in hESC

To test the hypotheses based of the data from methylation arrays, we focused on four Class B genes (frequently hypermethylated in cancer and hESC) widely reported to have tumour suppressor properties and that are frequently hypermethylated in cancer. We selected two (*MGMT* [O-6-methylguanine-DNA methyltransferase], *SLC5A8* [solute carrier family 5 (iodide transporter), member 8]) (Esteller et al., 2000; Li et al., 2003) from Class B-I (unmethylated in all NTT) and two (*PYCARD* [PYD and CARD domain containing], *RUNX3* [runt-related transcription factor 3]) (Conway et al., 2000; Li et al., 2002c) from Class B-II (unmethylated in a number of NTT). We first used bisulphite sequencing of multiple clones to determine accurately the promoter DNA methylation status of these genes in hESC and normal primary tissue (Fig. 4-8). In all cases, bisulphite sequencing data corroborated the array results and showed that hypermethylation in hESC affected the majority of CpG surrounding the transcription start-site of the genes selected. *MGMT* and *SLC5A8* showed dense promoter hypermethylation in hESC, but not in normal differentiated tissues (Fig. 4A, B, Fig. 5A, B, Fig. 6A, B), whilst Class B-II genes were frequently hypermethylated in hESC and sometimes in normal tissues (Fig. 7A, B, Fig. 8A, B). To better understand the role of promoter hypermethylation of these TSG in hESC and NTT, we used quantitative real time PCR (q-RT-PCR) to

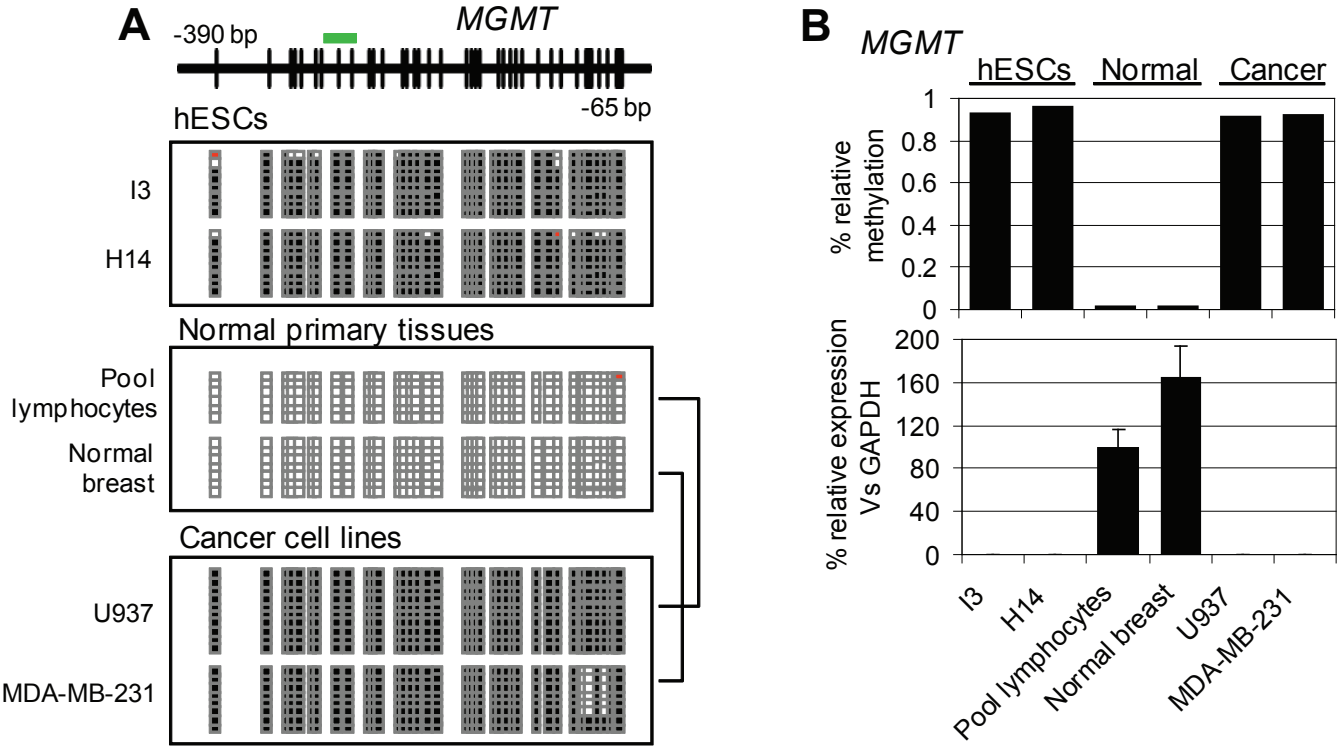


Figure 4. Promoter DNA hypermethylation and MGMT repression in hESC. (A) Bisulphite genomic sequencing of multiple clones of the MGMT promoter in hESC (I3, H14), NPT (pooled lymphocytes, normal breast) and two CCL of lymphoid and breast origin (U937 and MDA-MB-231, respectively). Black, methylated CpG; white, unmethylated CpG; red, CpG not present. The green bar above the diagram of the MGMT CpG island indicates the location of the probe used in the methylation arrays. (B) Relationship between MGMT promoter hypermethylation and expression in hESC, NPT, and CCL. We show the relative methylation signal obtained with methylation arrays (top) and MGMT mRNA expression levels relative to GAPDH (bottom).

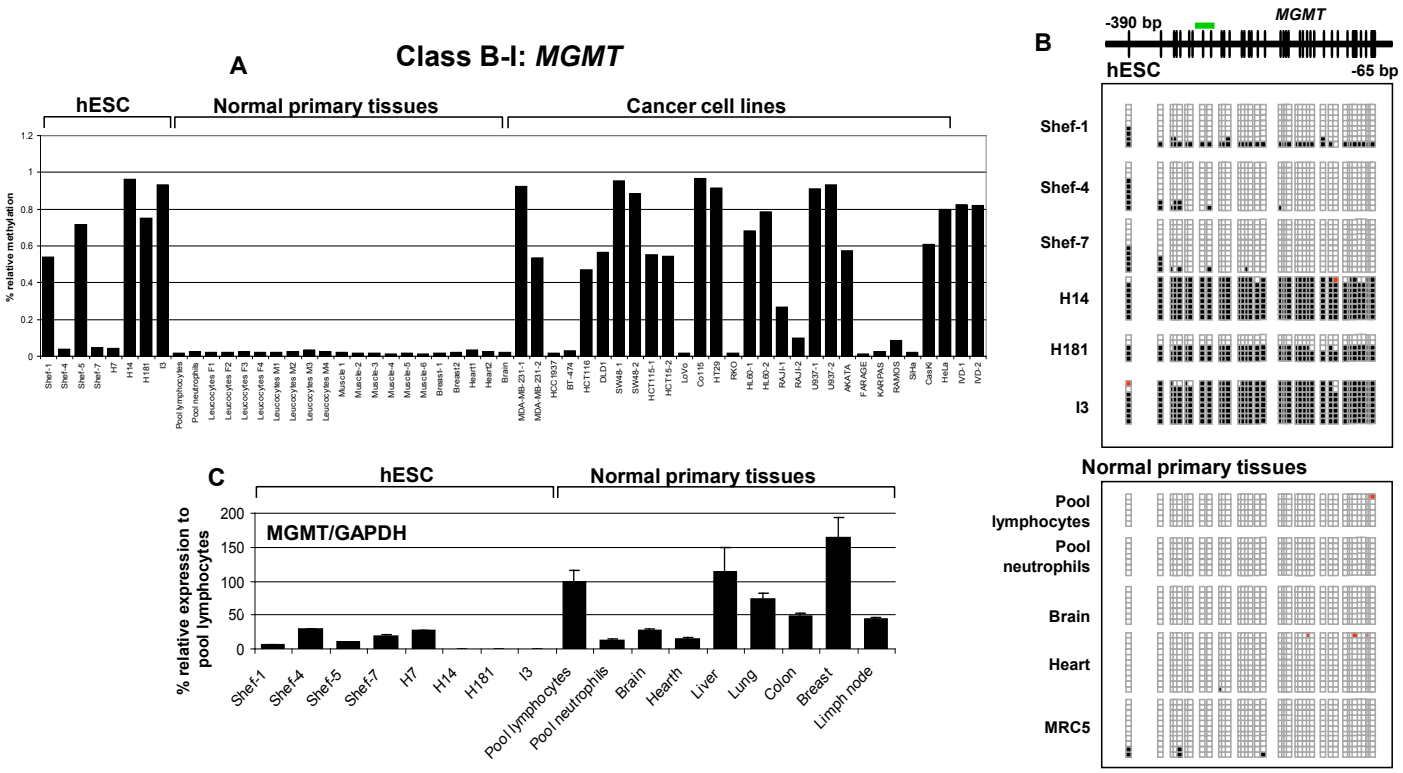


Figure 5. MGMT methylation status in hESC, NPT and CCL. (A) MGMT gene methylation profile obtained by Illumina arrays and expressed as relative methylation from fully unmethylated (0) to fully methylated (1). (B) Bisulphite genomic sequencing of multiple clones of the MGMT promoter in hESC and NPT. Colour code as for Fig. 4. (C) Relative expression of MGMT in hESC and NPT. q-RT-PCR data are normalised to GAPDH expression and presented as a percentage of normal lymphocytes (100%).

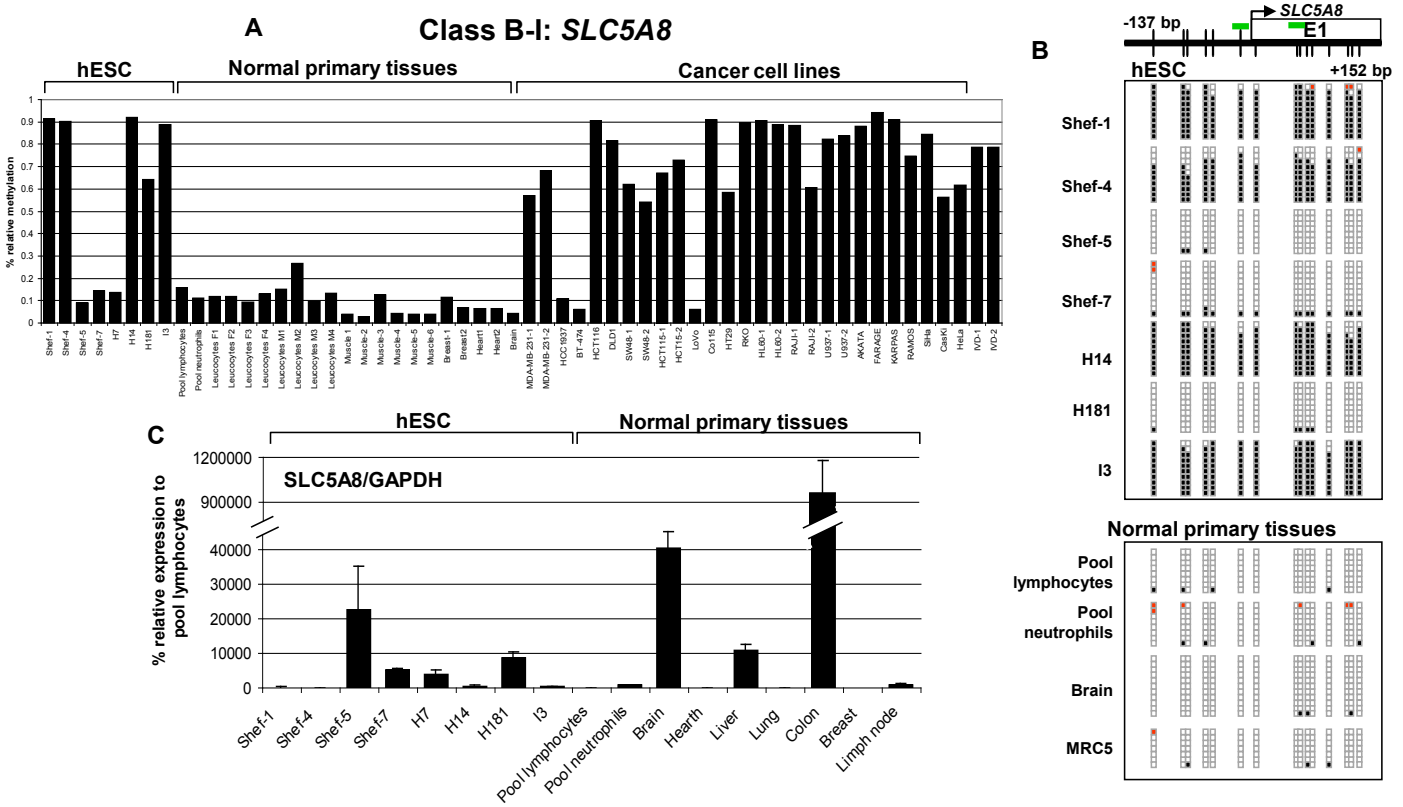


Figure 6. SLC5A8 hypermethylation in hESC. (A) SLC5A8 gene methylation profiles obtained by Illumina arrays and expressed as in Fig. 5. (B) Bisulphite genomic sequencing of multiple clones of the SLC5A8 promoter in hESC and NPT. Colour code as for Fig. 4. (C) Relative expression of SLC5A8 in hESC and NPT. q-RT-PCR data are normalised to GAPDH expression and presented as a percentage of normal lymphocytes.

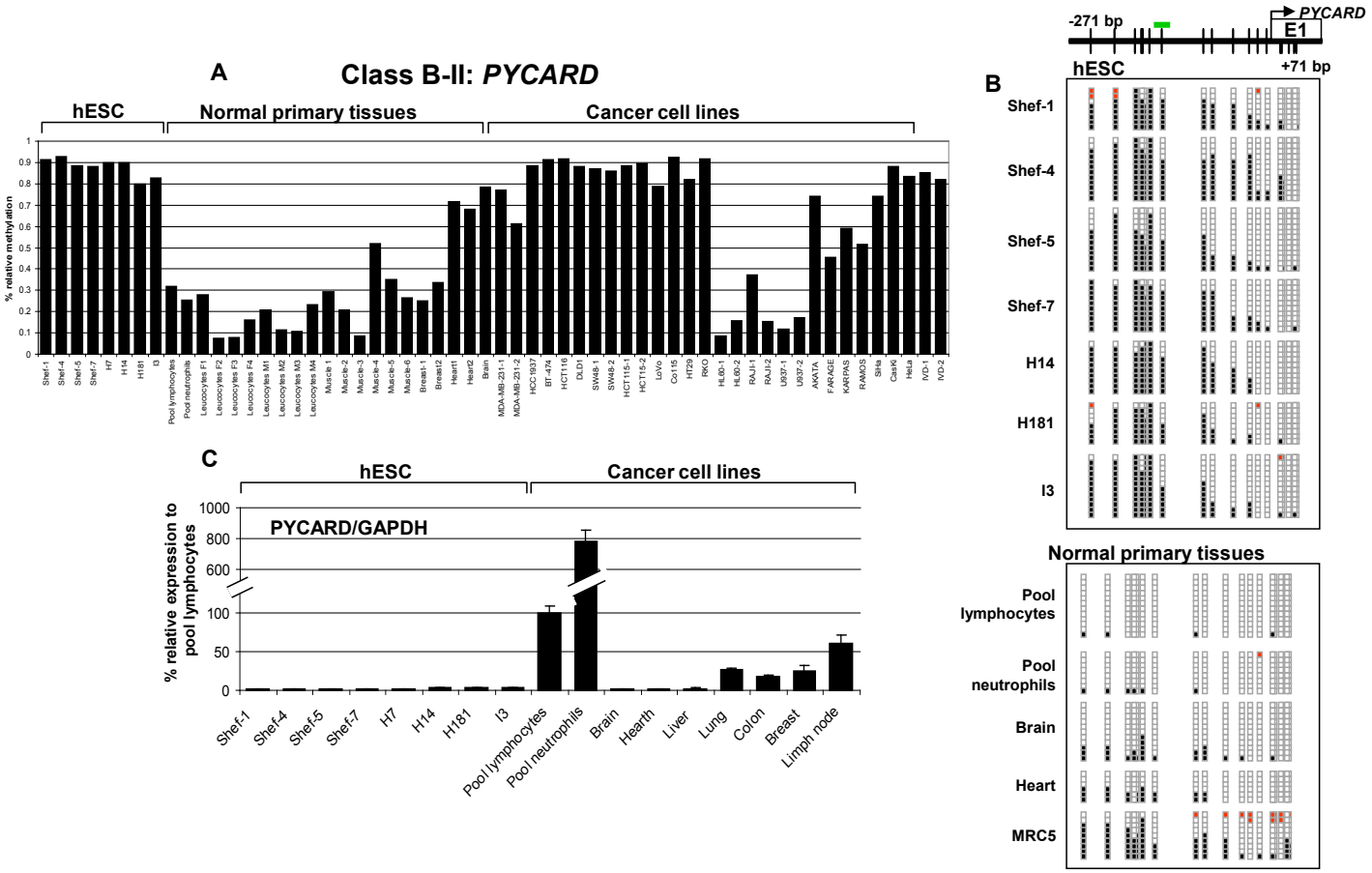


Figure 7. PYCARD hypermethylation in hESC. (A) PYCARD gene methylation profiles obtained by Illumina arrays and expressed as in Fig. 5. (B) Bisulphite genomic sequencing of multiple clones of the PYCARD promoter in hESC and NPT. Colour code as for Fig. 4. (C) Relative expression of PYCARD in hESC and NPT. q-RT-PCR data are normalised to GAPDH expression and presented as a percentage of normal lymphocytes.

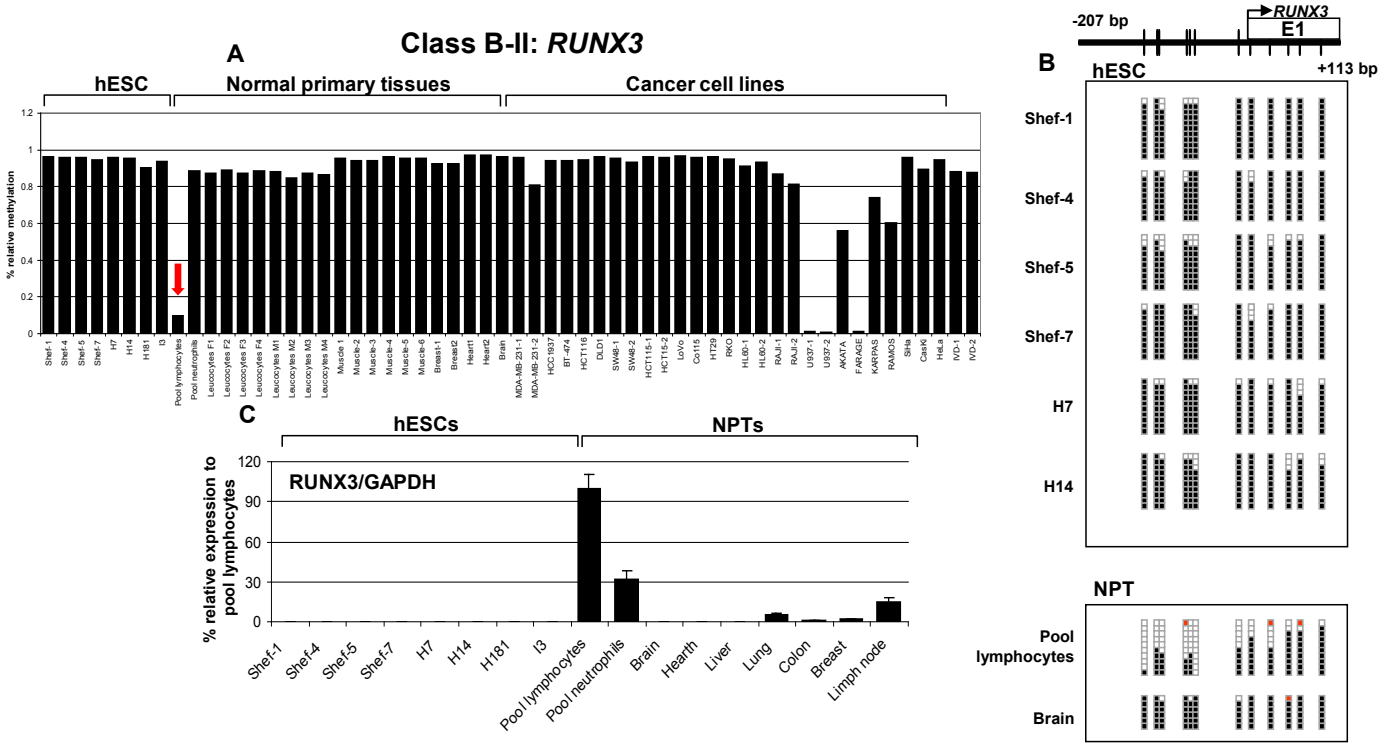


Figure 8. RUNX3 hypermethylation in hESC. (A) RUNX3 gene methylation profiles obtained by Illumina arrays and expressed as in Fig. 5. Red arrow indicates methylation levels in normal lymphocytes purified from blood. (B) Bisulphite genomic sequencing of multiple clones of the RUNX3 promoter in hESC and NPT. Colour code as for Fig. 4. (C) Relative expression of RUNX3 in hESC and NPT. q-RT-PCR data are normalised to GAPDH expression and presented as a percentage relative to normal lymphocytes

measure their expression in both sample groups (Fig. 4C, 5C, 6C, 7C, 8C).

Promoter hypermethylation was always associated with gene repression, but its absence in somatic primary tissues did not necessarily involve the upregulation of the gene; for example, whilst SLC5A8 was hypomethylated in all normal tissues analyzed (Fig. 6A), it was only overexpressed in brain, liver, and colon (Fig. 6B).

Loss of promoter hypermethylation and gene activation during hESC differentiation *in vitro*

To demonstrate further that hESC differentiation is associated with less DNA methylation at the promoter region

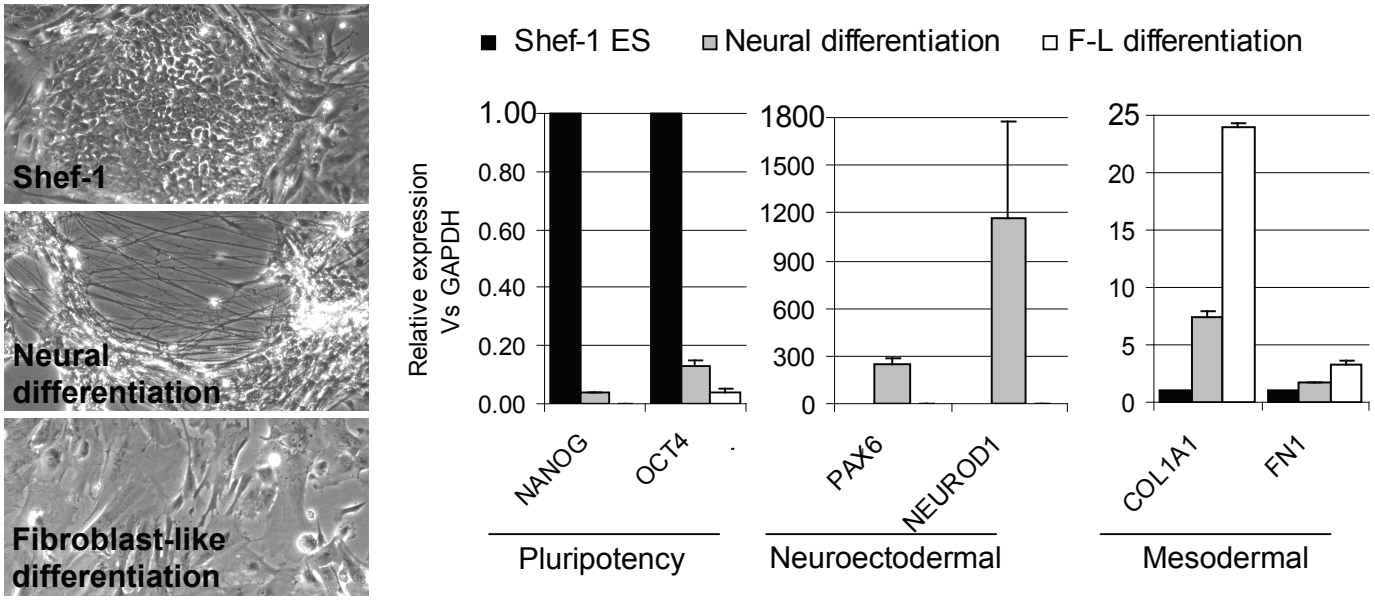


Figure 9. Human ESC differentiation in vitro. Left panels, Shef 1 stem cell line (top) and the same cells after neural differentiation (centre) and spontaneous differentiation to fibroblast-like cells (bottom). Right panels, relative mRNA levels of pluripotency (NANOG, OCT4), neuroectodermal (PAX6, NEUROD1), and mesodermal (COL1A1, FN1) markers before and after Shef-1 differentiation.

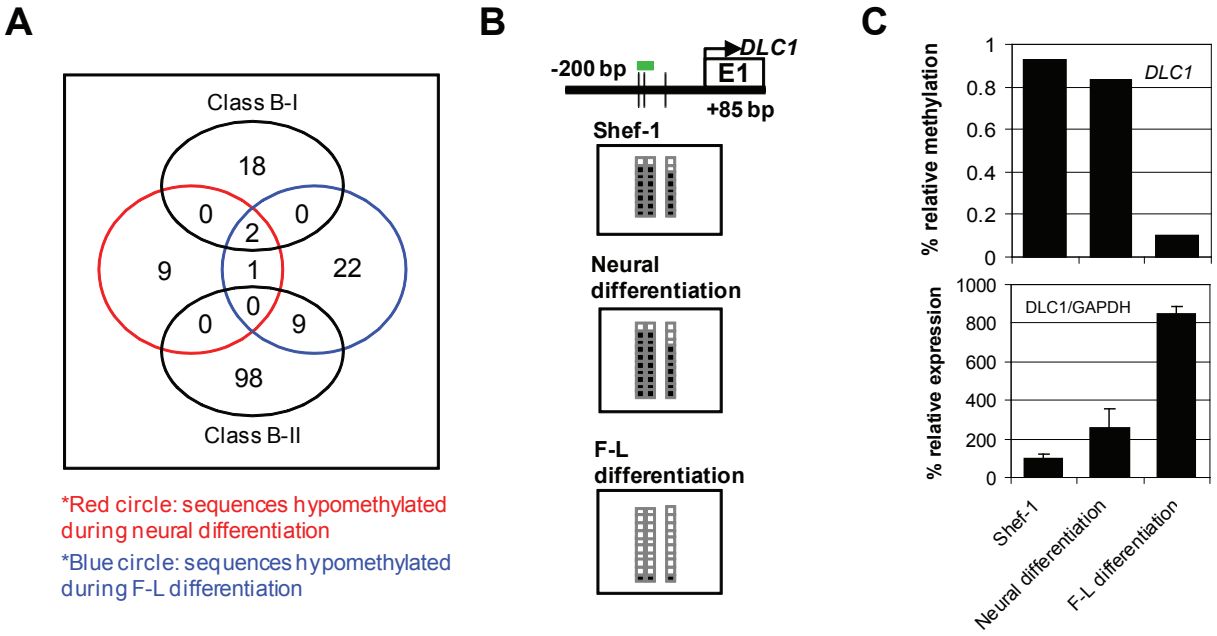


Figure 10. Loss of promoter DNA methylation during hESC differentiation in vitro. (A) Number of sequences hypomethylated during Shef-1 neural (red circle) and spontaneous (blue circle) differentiation, and their overlap with Class B-I and B-II genes (black circles). (B) Bisulphite genomic sequencing of multiple clones of the DLC1 promoter in Shef-1 stem cell line (top), and the same cells after neural differentiation (centre) and spontaneous differentiation to fibroblast-like cells (bottom). The colour code is as for Figure 4. (C) Relationship between DLC1 promoter hypermethylation and expression during Shef-1 differentiation. Relative methylation signal obtained with the methylation arrays (top) and DLC1 mRNA expression levels relative to GAPDH (bottom).

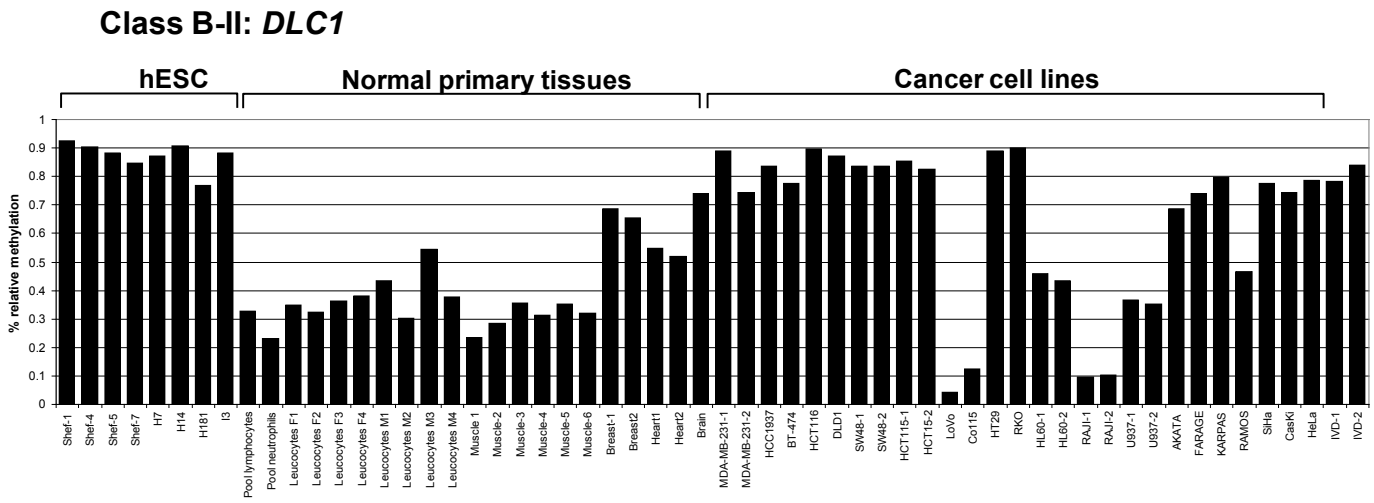


Figure 11. DLC1 hypermethylation in hESC. DLC1 gene methylation profiles obtained by Illumina arrays and expressed as relative methylation, from fully unmethylated (0) to fully methylated (1).

of certain genes, we induced *in vitro* differentiation of the hESC line Shef-1 in two cell lineages (fibroblast-like cells [F-L] and neural precursors; Fig. 9A). We assessed lineage specification using previously published markers (Cai et al., 2006) (Fig. 9A, right), then used methylation arrays to identify genes that were hypomethylated during differentiation. We found that 12.98% (37/285) of the genes hypermethylated in Shef-1 (which were not methylated in all NTT analysed) were demethylated during *in vitro* differentiation. Of these, 12 genes were demethylated during neuron differentiation and 25 during F-L differentiation (Table S6). Three of these genes were common to both groups (Fig.10A), two of which belonged to Class B-II. Whilst 9/25 of the genes demethylated during F-L differentiation were Class B-II, none of the 12 genes demethylated during neuron differentiation belonged to this category. To show that some TSG that are frequently hypermethylated in cancer and hESC can lose methylation during differentiation, we focused our attention on *DLC1 (*deleted in liver cancer 1), chosen because the methylation arrays showed that it lost promoter methylation during F-L differentiation of Shef-1, and because it is a TSG that is frequently hypermethylated in cancer (Fig. 11) (Ying et al., 2007; Yuan et al., 2003).

Bisulphite sequencing of multiple clones corroborated methylation array results and showed that the *DLC1* promoter is hypermethylated in Shef-1 and is demethylated during spontaneous, but not neural, differentiation (Fig. 10B). In q-RT-PCR experiments, DLC1 was poorly expressed in the Shef-1 cells and was overexpressed during spontaneous, but not neural differentiation (Fig. 10C).

TSG repressed by promoter hypermethylation in haematopoietic stem cell progenitors

Having shown that some cancer genes are hypermethylated and repressed in hESC, and that they can lose methylation during hESC differentiation *in vitro*, we studied whether this phenomenon is restricted to embryonic development or, conversely, is an epigenetic mechanism associated with stemness status regardless of the cell ontogenetic stage. We used methylation arrays to identify genes hypermethylated in $CD34^+$ somatic stem cell progenitors compared to peripheral blood lymphocytes and neutrophils, two types of adult primary cells derived from $CD34^+$ haematopoietic progenitors. We identified 362 hypermethylated sequences in $CD34^+$ cells (array signal > 0.7) (Table S7).

The great majority of these sequences (92.27%, 334/362) were also methylated in hESC, and most of these sequences were frequently hypermethylated in CCL (83.43%, 302/362) (Fig. 12A; Table S6). These results suggest that the hypermethylation of cancer genes can occur in stem cells regardless of ontogenetic stage (embryo vs. adult). We next identified nine sequences that were hypermethylated in $CD34^+$ cells relative to peripheral lymphocytes, and 16 sequences that were hypermethylated in these progenitor cells relative to neutrophils (Table S8). Most of the sequences identified were also frequently hypermethylated in hESC (8/9 for lymphocytes, 14/16 for neutrophils) and CCL (6/9 for lymphocytes, 13/16 for neutrophils). There were no sequences common to lymphocytes and neutrophils; most of these sequences and were occasionally hypermethylated in NPT. Of these sequences, 28 had previously been classified as Class B-II genes, whilst none was from Class B-I (Fig. 12A).

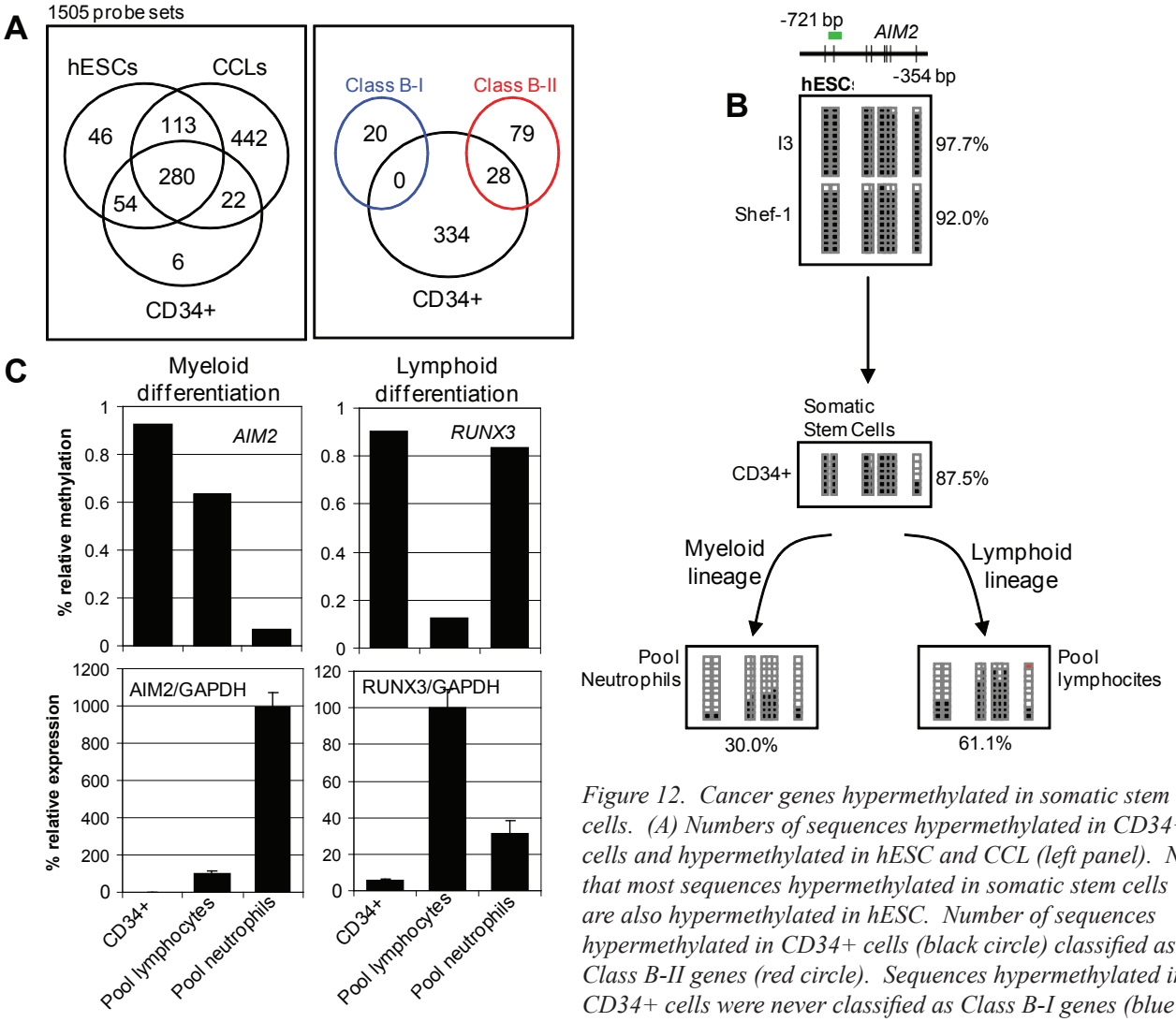


Figure 12. Cancer genes hypermethylated in somatic stem cells. (A) Numbers of sequences hypermethylated in CD34+ cells and hypermethylated in hESC and CCL (left panel). Note that most sequences hypermethylated in somatic stem cells are also hypermethylated in hESC. Number of sequences hypermethylated in CD34+ cells (black circle) classified as Class B-II genes (red circle). Sequences hypermethylated in CD34+ cells were never classified as Class B-I genes (blue circle) (right panel). (B) Bisulphite genomic sequencing of multiple clones of the AIM2 promoter in Shef-1 and I3 stem cell lines (top), CD34+ haematopoietic stem cell progenitors (centre), and terminally differentiated haematopoietic cells (peripheral lymphocytes and neutrophils; bottom). Colour code as for Fig. 4. (C) Relationship between AIM2 and RUNX3 promoter hypermethylation and expression in CD34+ cells and terminally differentiated haematopoietic cells (peripheral lymphocytes and neutrophils). Top panels, relative methylation signal in the methylation arrays; bottom panels, AIM2 and RUNX3 mRNA expression levels relative to GAPDH.

Finally, to demonstrate that some cancer hypermethylated genes are also frequently hypermethylated in somatic progenitor stem cells and that their methylation is important for lineage specification, we evaluated two genes, *RUNX3* and *AIM2*. RUNX3 was selected because, in accordance with published data (Kang et al., 2007), our methylation arrays showed that relative to $CD34^+$ cells, *RUNX3* was hypomethylated in peripheral lymphocytes and less so in peripheral neutrophils. *AIM2* was chosen as it is frequently hypermethylated in cancer (Fig. 13) (Woerner et al., 2007) and because, unlike *RUNX3*, it is demethylated specifically in the myeloid lineage (Fig. 11B, C). The bisulphite sequencing data confirmed the array results, showing that $CD34^+$ cells and peripheral lymphocytes were densely methylated at the *AIM2* promoter, whilst the peripheral neutrophils were almost unmethylated (Fig. 12B). To determine the role of *AIM2* and *RUNX3* promoter hypermethylation in haematopoietic differentiation, we used qRTPCR to analyze their expression in our groups of samples (Fig. 12C). In both genes, promoter hypermethylation was always associated with gene repression, and loss of promoter methylation in *AIM2* and *RUNX3* was associated with their re-expression in peripheral lymphocytes and neutrophils, respectively (Fig. 4C).

Whole genome promoter DNA methylation analysis by Infinium Array

To extend our analysis of promoter methylation changes during cell differentiation, we used the more complete HumanMethylation27 BeadChip Infinium Methylation Array, which provides quantitative methylation measurement at 27,578 CpG loci covering 14,495 gene promoters or regulatory regions. This includes a large part of the genes characterised in

the human genome that possess CpG in their promoter both at high and at low density (Table S9).

We compared the DNA methylation status of 10 distinct undifferentiated hESC lines and 10 differentiated normal tissues (1 brain, 3 colon, 2 sperm, 2 total blood, 1 lymphocyte sample and 1 neutrophil sample), and focused analysis on genes that undergo demethylation during differentiation (Table S10). Again, due to the different origin of each sample, we excluded sex chromosome-linked genes to avoid interference of Xinactivation-dependent methylation. We identified 7513 sequences corresponding to 4962 genes (35% of the total array) hypermethylated in stem cells (array signal > 0.7 in ≥ 40% (4/10) of the samples). Here we used stricter criteria, because the larger number of starting genes would yield gene populations in numbers suitable for statistical analysis. Of these hESC hypermethylated sequences, we identified 1954 sequences, corresponding to 1582 distinct gene promoters, that become unmethylated (array signal < 0.3) in at least one normal tissue. This corresponds to 11.4% (1585/13890) of the total genes in the array and to 31.9% (1585/4962) of all genes hypermethylated in stem cells. After performing gene ontology analysis through the DAVID interface, we observed that biological function and cellular localisation of gene products are not distributed stochastically. Biological processes concerning immune-related functions such as immune response, defence response to bacteria, and inflammatory response are strongly enriched, as is cellular localisation in the plasma membrane or extracellular region (Table 3). Many of these genes seem to have a characterised function in the definition of the mesodermal-derived tissues, such as the immune system and muscle. Moreover, many of these genes have a signal peptide and are localised in the external part of the membrane or are secreted. Eighty percent (1268/1585) of the probes are classified by the Illumina array spreadsheet as false CpG islands, meaning that the promoters have a low CpG density in the region surrounding that of interest. Loss of methylation during differentiation *in vivo* to normal tissue thus appears to be quite a frequent feature in the genome.

Whole genome DNA methylation analysis of *in vitro* differentiation to mesodermal lineage

To expand our analysis of DNA methylation in *in vitro* differentiation, we specifically analysed promoter demethylation in Shef-1 differentiation to a mesodermic lineage, as we had with the Goldengate platform. Here we compared Shef-1 undifferentiated ES cells and their differentiated counterpart, fibroblast-like cells (F-L), selecting probes whose array methylation signal shows >40% change between the two samples (Table S11). We found that 217 probes, corresponding to 202 genes, are demethylated specifically in this lineage differentiation, corresponding to 12.7% (202/1575) of the genes previously identified as demethylated during differentiation to normal tissue. Only 25 genes were found to be hypermethylated during this process. Gene ontology processing showed that these genes are enriched in the same biological function and cellular localisation as the genes discussed above, which encode mainly membrane and extracellular proteins related to immune system function (Table 4). We also found genes, transcription factors or chromatin-regulating genes involved in regulation of mesodermal-immune differentiation, such as *RUNX1* and *HDAC9*, in addition to the previously described

GO TERM (Biological process)	Nº of genes	p value
GO:0006955~immune response	196	1.04E-48
GO:0006952~defense response	157	4.48E-30
GO:0042742~defense response to bacterium	35	8.95E-12
GO:0006954~inflammatory response	73	7.53E-11
GO:0009617~response to bacterium	49	1.71E-10
GO TERM (Cell component)		
GO:0005576~extracellular region	301	1.12E-17
GO:0005886~plasma membrane	464	2.50E-14
GO:0005887~integral to plasma membrane	202	9.08E-12
GO:0031226~intrinsic to plasma membrane	203	2.51E-11
GO:0044459~plasma membrane part	312	7.84E-10

Table 3. GO terms significantly enriched in hESC hypermethylated genes undergo demethylation during in vivo differentiation.

GO TERM (Biological process)	Nº of genes	p value
GO:0006955~immune response	24	3.43E-06
GO:0006952~defense response	21	2.69E-05
GO:0042742~defense response to bacterium	9	3.97E-05
GO:0009617~response to bacterium	11	6.83E-05
GO:0002684~positive regulation of immune system process	12	7.37E-05
GO TERM (Cell component)		
GO:0005887~integral to plasma membrane	29	4.52E-04
GO:0005576~extracellular region	42	5.04E-04
GO:0031226~intrinsic to plasma membrane	29	6.34E-04
Uniprot sequence feature		
disulfide bond	70	4.00E-13
glycosylation site:N-linked (GlcNAc...)	82	2.33E-10
signal peptide	71	2.36E-10

Table 4. GO terms significantly enriched in genes demethylated during in vitro differentiation to F-L.

DLC1. Comparison of our gene list with published data on promoter occupation by the PRC2 subunit Suz12 (Lee et al., 2006a) and on the presence of the bivalent mark (Zhao et al., 2007) showed that only 11 genes (0.6%) are PRC2 targets in hESC cells, and only 6 (0.3%) display a bivalent mark, whereas 137 (68%) showed neither of the two marks (trimethyl-K4 and trimethyl-K27). There is thus a certain percentage of late developmental genes, most with a function in mesodermal lineage and/or the immune system, that are repressed in stem cells whose promoter, even if not CpG-dense, is hypermethylated in embryonic stem cells; other repression marks like Polycomb repression and the bivalent mark are mostly absent. These genes can be demethylated during differentiation both *in vivo* and *in vitro*, and this phenomenon is likely to be related to the regulation of their expression in mesodermal-immune system differentiated cells.

DNA methylation alteration due to prolonged hESC culture *in vitro*

Using this larger Infinium platform, we were interested in further quantitative analysis of how *in vitro* maintenance of hESC alters the epigenetic status in terms of promoter methylation. Previous reports showed that some genes can be hypermethylated after prolonged stem cell culture *in vitro*, but that these changes were not extensive and appeared to be cell line-dependent rather than a common feature (Allegrucci et al., 2007; Maitra et al., 2005). hESC cultured for an large passage number are often subject to a phenomenon termed culture adaptation, that implies many genetic, phenotypic, and biochemical changes. We analysed promoter DNA methylation from two pairs of cell lines, at low and high passage number (Table S12). The original low passage cells showing a "normal" phenotype were H7S14 and H14S9, and their "adapted" counterparts H7S6 and H14BJI; both normal clones are estimated to be at passage ~30-35, while adapted clones are cultured for more than 100 additional passages (Baker et al., 2007; Enver et al., 2005). We found that 205 genes were hypermethylated (array signal difference > 40%) and 67 genes were demethylated in adapted compared to normal H7 cells; similarly, 197 genes were hypermethylated and 153 genes demethylated in adapted compared to normal H14 cells. This indicates that in both cell lines, ~2% of the genes in the Infinium platform are subject to alterations in DNA methylation. Only 34 genes (~15%) are commonly hypermethylated in these two cell lines, and only 3 are demethylated in both; there is thus apparently a low degree of coincidence in the aberrant mechanisms that lead to promoter hypermethylation. Gene ontology analysis for these gene groups showed that both hypermethylated genes in adapted vs. normal H7 and H14 cells are significantly enriched for transcription factor containing a Zn^{++} finger domain (in particular C2H2; 17 for H7 and 44 for H14; Table 5). This probably indicates that this protein family is prone to hypermethylation during prolonged hESC culture *in vitro*. Demethylated genes showed no tendency toward a specific gene ontology term.

	H14		H7	
GO TERM (Biological process)	genes	p value	genes	p value
GO:0006350~transcription	52	1.38E-15	32	1.29E-04
GO:0034961~cellular biopolymer biosynthetic process	55	2.31E-13	34	1.72E-03
GO:0043284~biopolymer biosynthetic process	55	2.70E-13	34	1.83E-03
GO:0045449~regulation of transcription	51	3.65E-11	33	2.62E-03
GO TERM (Cell component)				
GO:0008270~zinc ion binding	52	4.22E-13	30	3.82E-04
GO:0003677~DNA binding	49	4.58E-11	33	3.26E-03
UNIPROT SEQUENCE FEATURE				
zinc finger region:C2H2-type 8	40	5.39E-34	14	6.54E-06
zinc finger region:C2H2-type 7	40	1.01E-32	14	1.47E-05
zinc finger region:C2H2-type 10	36	4.69E-32	14	6.04E-07

Table 5. GO terms significantly enriched in genes hypermethylated during hESC culture adaptation.

PART II: Histone modification in hESC differentiation and developmental gene activation

Global histone H4 acetylation increase during differentiation

To study new pathways in chromatin regulation during stem cell differentiation, we began with analysis of histone modifications during *in vitro* differentiation. As described in the introduction, a specific histone mark signature characterises hESC, based on methylation of histone H3 (methylated at K4 and K27 the bivalent domain) (Bernstein et al., 2006). Nevertheless, there are few data on histone acetylation, an important modification in the regulation of gene expression. We first studied global acetylation of histone H4 by HPLC purification of total H4, and top-down mass spectrometry of this purified histone (Fig. 14). We found that the profile of the H4 sample from Shef-1 ES differed from that of EB after 14 days of differentiation. The most relevant difference was observed in the peaks corresponding to the mono- and diacetylated forms of this histone, which appear higher in the EB. Acetylation on H4 is thought to occur sequentially (Clarke et al., 1993; Turner, 1989) and involves residues K16, K12 and K8/K5; H4K16 is thus probably responsible for the difference observed. This residue is specifically deacetylated by SirT1, an enzyme implicated in differentiation of several cell types; we therefore examined whether SirT1 has a role in hESC differentiation.

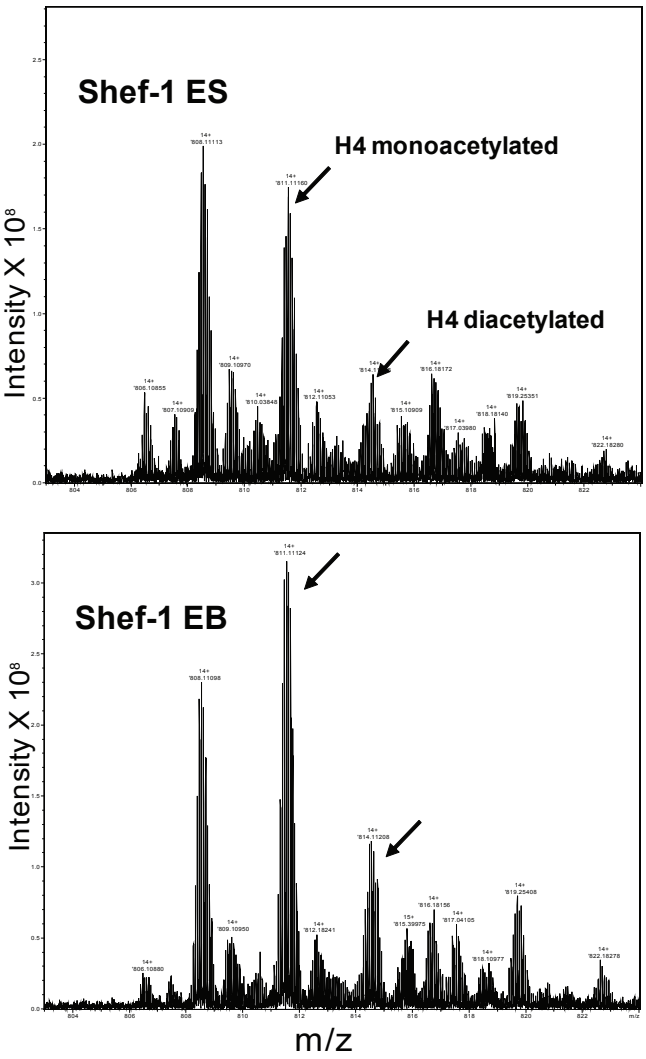


Figure 14. Top-down mass spectrometry analysis of HPLC-purified histone H4 in Shef-1 cells, undifferentiated (ES) and differentiated to embryo bodies for 14 days (EB). Peaks represent signal intensity at a defined mass-to-charge (m/z) ratio. Higher peaks represent distinct acetylation levels (arrows indicate mono- and diacetylated forms), while lower peaks are probably due to other modifications, particularly methylation.

SirT1 downregulation during hESC differentiation

To study the putative role of SirT1 in hESC differentiation, we measured SirT1 mRNA levels during the course of *in vitro* differentiation of the Shef-1 and H-181 hESC lines. Withdrawal of basic fibroblast growth factor (bFGF) and feeder cells causes spontaneous differentiation of both of these lines into embryonic bodies (EB) (Li et al., 2009). At 15 days after the induction of differentiation, SirT1 mRNA levels were 60% lower in Shef-1 cells and 50% lower in H-181 cells (Fig. 15, top). Consistent with this, there was a marked reduction in SirT1 protein levels in 15-day EB compared to hESC (Fig. 15, bottom), in accordance

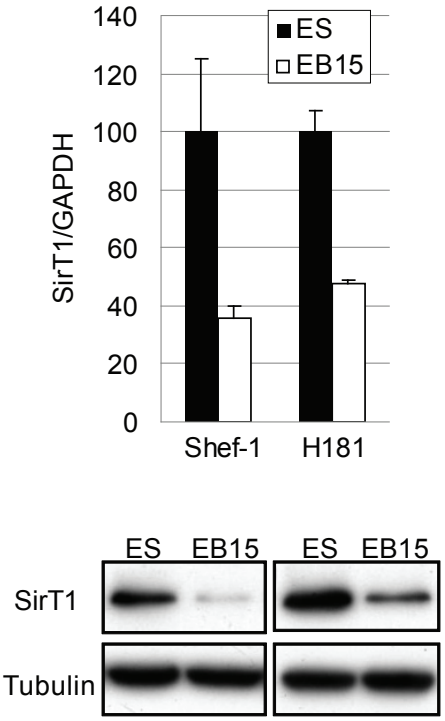
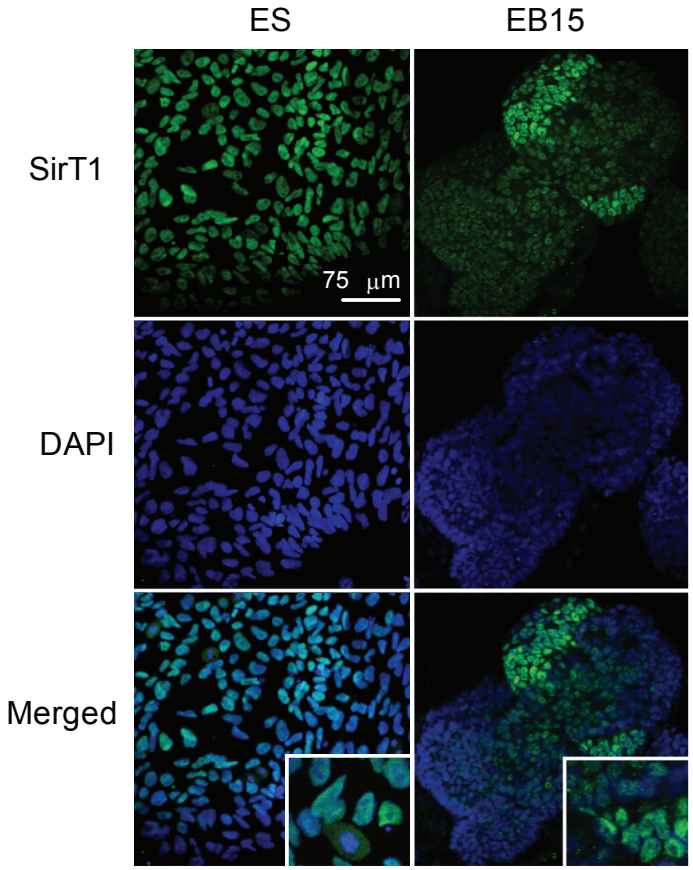


Figure 15. Downregulation of SirT1 during hESC differentiation. q-RT-PCR analyses of SirT1 mRNA levels in hESC (ES) cells and 15-day EB (EB15) from Shef-1 and H-181 lines (top). Results are represented as the amount of SirT1 mRNA relative to control GAPDH mRNA. Western blot analysis of the same samples (bottom) using anti-SirT1 antibody and anti-α-tubulin as loading control.



with previous findings in mice (Kuzmichev et al., 2005). To further characterise SirT1 downregulation during hESC differentiation, we performed immunofluorescence staining of SirT1 in hESC and 15-day EB. SirT1 staining in hESC was mostly nuclear except during mitosis, when it was diffused in the cytoplasm (Fig. 16). In 15-day EB, we found lower SirT1 staining levels and heterogeneous distribution within different cell populations (Fig. 16). To characterise precisely the downregulation of SirT1 during hESC differentiation, we assessed RNA and proteins levels in a time-course experiment during spontaneous differentiation of Shef-1 cells into EB and subsequently into fibroblast-like cells (F-L cells) (Fig. 17A). F-L cells were obtained by inducing EB attachment, followed by culture for a further 15 days (Li et al., 2009). SirT1 mRNA levels decreased gradually during hESC differentiation, whilst SirT1 protein levels dropped markedly only 7 days after induction of differentiation, and remained low throughout the process (30 days) (Fig. 17B); this suggests that SirT1 regulation during hESC differentiation might occur at several levels.

Figure 16. SirT1 immunostaining during hESC differentiation. Confocal fluorescence microscopy assay showing the cellular localization of SirT1 (green) in Shef-1 undifferentiated hESC and 15-day EB. Nuclei are stained with DAPI (blue).

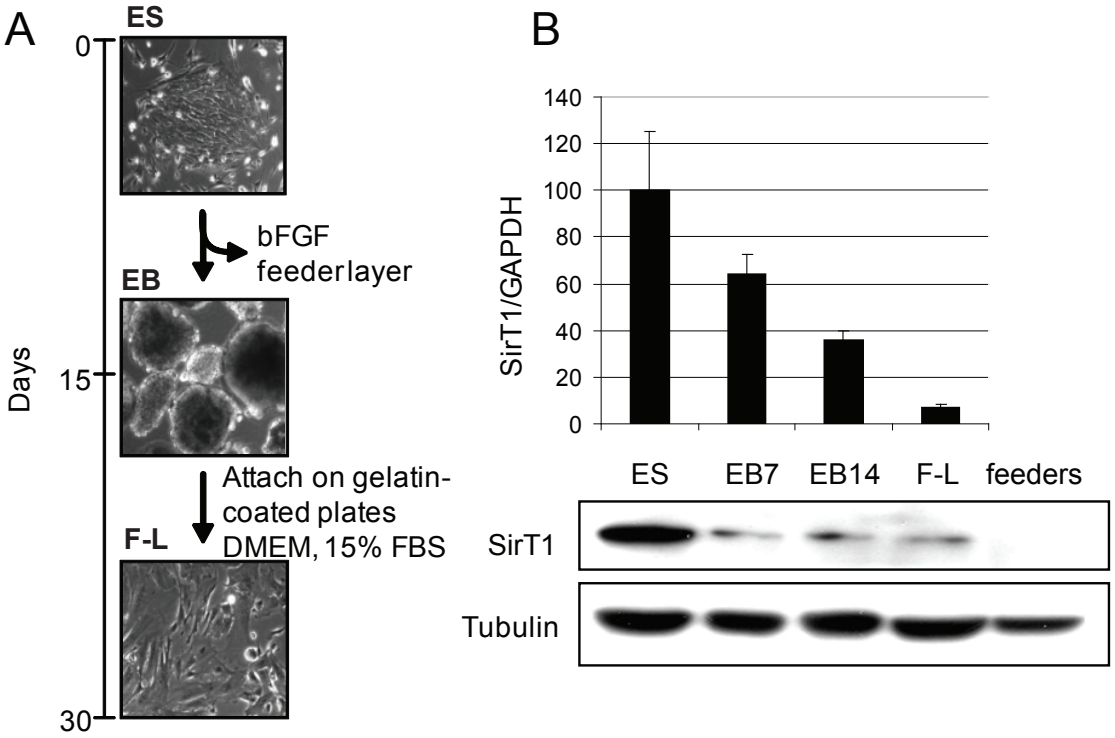


Figure 17. Time-course of SirT1 downregulation during hESC differentiation. (A) Flowchart of in vitro hESC differentiation . Representative images of hESC (ES), EB and F-L cells were obtained by phase-contrast microscopy. hESC differentiated to EB after bFGF withdrawal and growth in suspension for 15 days. F-L cells were obtained by attachment of EB on gelatin-coated plates and culture in DMEM + 15% FBS. (B) Time-course expression study of SirT1 by q-RT-PCR (relative to GAPDH) and WB during hESC Shef-1 differentiation. SirT1 is not detected in feeder cells (mitomycin C-treated MEF), which could contaminate the hESC sample.

SirT1 mRNA stabilisation through CARM1-dependent HuR methylation

To study the molecular mechanisms involved in SirT1 downregulation during hESC differentiation, we first tested a number of potential ways to regulate SirT1 expression, including SirT1 promoter epigenetic status and activity. As the SirT1 promoter contains a large CpG island, DNA methylation is a likely candidate for the regulation of its expression. We observed that SirT1 promoter is unmethylated in all conditions studied: in hESC Shef-1 and H-181 as well in differentiated EB from these cell lines, and in a differentiated tissue (PBL lymphocytes) (Fig. 18).

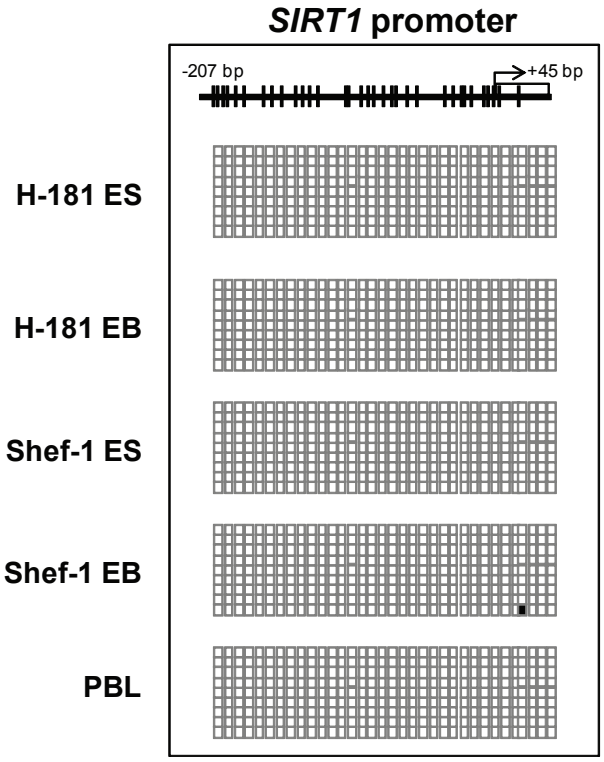
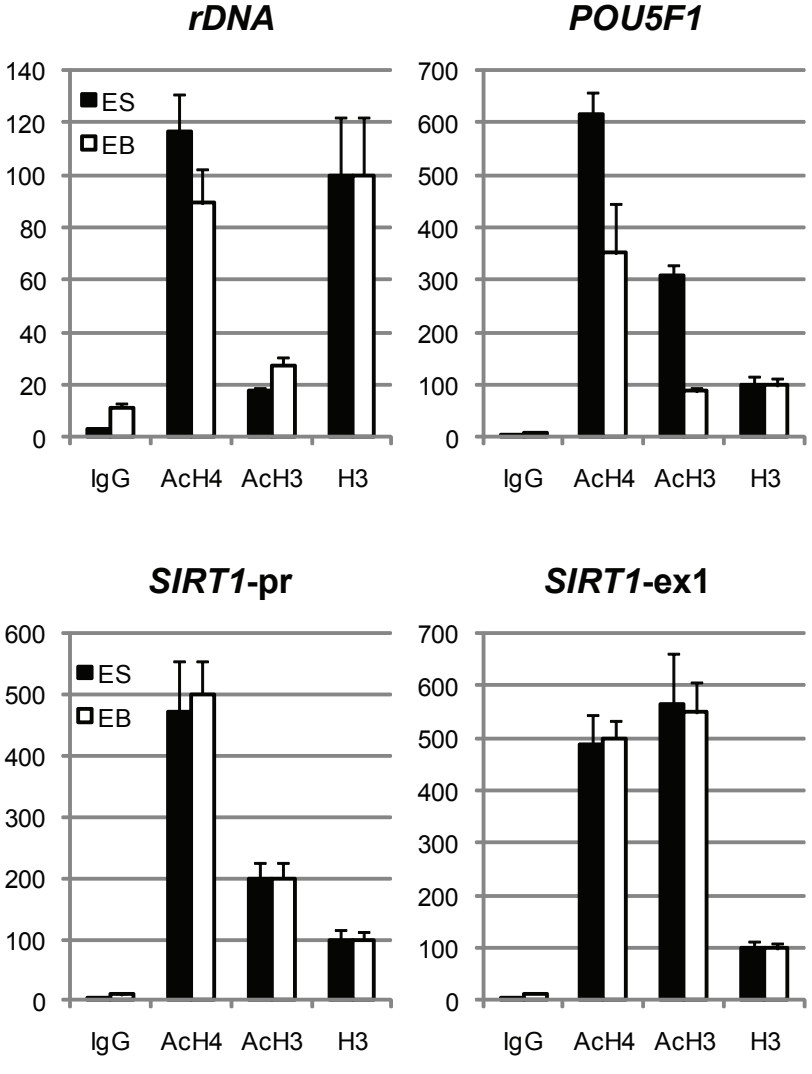


Figure 18. Methylation status of the SirT1 promoter. Bisulphite genomic sequencing of multiple clones of the SirT1 promoter in two hESC lines (H-181, Shef-1), the corresponding differentiated sample (EB), and peripheral blood lymphocytes (PBL). Black box, methylated CpG; white box, unmethylated CpG.



Transcriptional activity of a gene promoter can be evaluated by different methods, including the binding of RNA-Pol II, the presence of activation marks such as trimethyl-H3K4 or chromatin condensation marks such as acetylation of histones H3 and H4. The first two possibilities were discarded in the case of embryonic stem cells, as it has been reported that RNA-pol and trimethyl-H3K4 are detectable even in inactive gene promoters in hESC (Efroni et al., 2008; Guenther et al., 2007). We performed a q-ChIP assay (Fig. 19) for global acetylation as a marker of promoter activation in two regions of the SirT1 promoter, and validated its suitability using a gene whose expression is negatively regulated during

Figure 19. SirT1 promoter activity. q-ChIP of global acetylated histone H4 and histone H3 in Shef-1 hESC and EB. q-PCR corresponding to promoters of rDNA as a negative control, POU5F1 (OCT4) as a positive control of downregulation of activation markers in differentiation, and two regions of the SirT1 promoter located up- and downstream of the transcription start site. Final results are expressed as the percentage of bound/unbound ratio of DNA copy number for each IP, further normalised with respect to total H3 enrichment, considered constant in chromatin.

differentiation (POU5F1, Oct4) as well as an unregulated gene (rDNA, which encodes the rRNA precursor). Whereas promoter acetylation of POU5F1 decreased notably during differentiation, neither of the SirT1 regions selected appeared to be deacetylated. We therefore concluded that the main regulation of SirT1 expression during differentiation is not at the promoter level.

As SirT1 mRNA is first downregulated, once we excluded the mRNA synthesis rate, the other possibility for regulation of mRNA levels is mRNA degradation. In cancer cells, SirT1 mRNA stability can be regulated by the RNA-binding protein HuR (Abdelmohsen et al., 2007), which also has a role in cell differentiation (Figueroa et al., 2003); we thus hypothesised that HuR regulates SirT1 RNA stability during hESC differentiation. To test this, we examined the stability of SirT1 RNA by treating cells with actinomycin D to inhibit *de novo* transcription. We monitored levels of SirT1 mRNA and the housekeeping control GAPDH mRNA by q-RT-PCR, and found that SirT1 mRNA was notably less stable in differentiating EB than in pluripotent hESC (Fig. 20). GAPDH mRNA, which is not a HuR target (Lopez de Silanes et al., 2004), showed no differences between hESC and EB. To determine whether the decrease in SirT1 stability was due specifically to HuR, we measured the amount of SirT1 mRNA bound to HuR during hESC differentiation, using immunoprecipitation (IP) assays with an anti-HuR antibody in conditions that preserved HuR-mRNA complex composition and by monitoring RNA using q-RT-PCR. There was a clear decrease in the amount of SirT1 RNA bound to HuR in 15-day EB compared to hESC (Fig. 21A), which suggests that the decrease in SirT1 RNA during hESC differentiation is mediated by loss of HuR/SirT1 mRNA binding.

To confirm SirT1 regulation by HuR, we depleted HuR in the Shef-1 hESC line with siRNA. HuR silencing resulted in a marked reduction of SirT1 (Fig. 21B), further evidence of HuR-mediated regulation of SirT1 mRNA stability in hESC. HuR binding to β-catenin, another independent HuR target, also decreased during hESC differentiation (Fig. 22), suggesting that global HuR activity is also regulated during hESC differentiation.

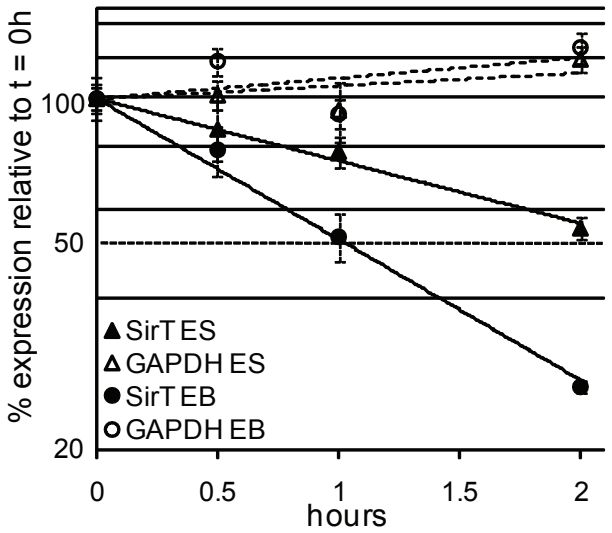


Figure 20. mRNA stability assay. q-RT-PCR of SirT1 and GAPDH in Shef-1 hESC (ES) and EB at 3 days of differentiation, after actinomycin D treatment and collection at the times indicated. Data are relative to mRNA levels at t = 0 h for each sample.

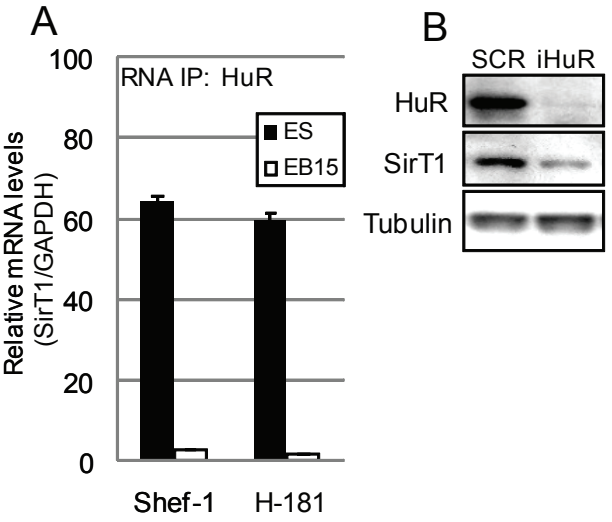


Figure 21. SirT1 mRNA binds by HuR. (A) RNA immunoprecipitation of HuR in hESC (ES) and EB differentiated for 15 days (EB15) of Shef-1 and H-181 hESC lines. SirT1 levels were detected by q-RT-PCR (relative to GAPDH). (B) HuR interference. Western blot of HuR, SirT1 and α-tubulin in Shef-1 cells, 3 days post-transfection with control siRNA (SCR) and HuR-specific siRNA (iHuR).

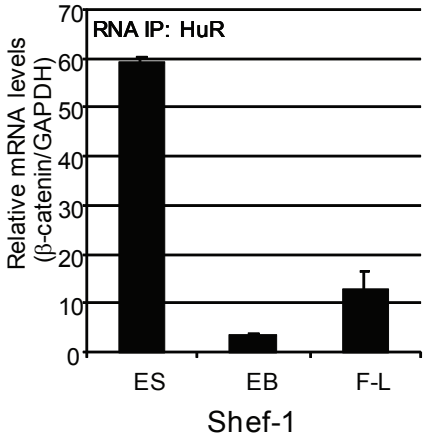


Figure 22. HuR binding to β-catenin RNA. RNA immunoprecipitation of HuR in hESC (ES), EB differentiated for 15 days (EB15) and fibroblast-like cells (F-L) of Shef-1 and H-181 hESC lines. β catenin levels were detected by q-RT-PCR (data relative to GAPDH).

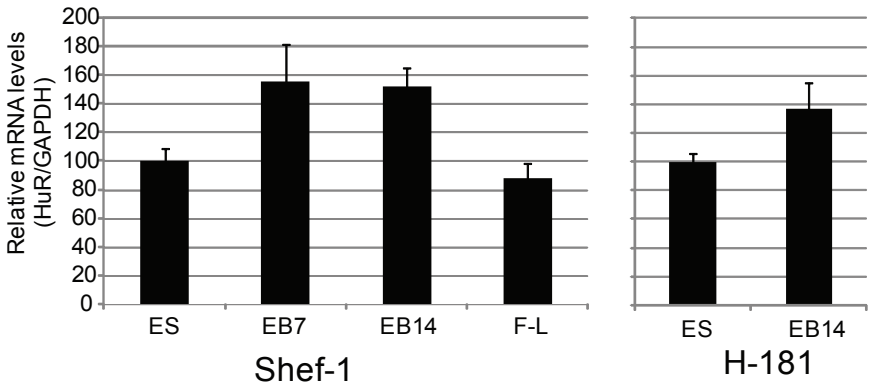


Figure 23. HuR mRNA expression. Time-course expression study of HuR by qRT-PCR (relative to GAPDH) in hESC Shef-1 (ES), Shef-1 EB differentiated for 7 and 14 days (EB7 and EB14, respectively), and for 30 days to fibroblast-like cells (F-L), H-181 hESC and EB.

To test this possibility, we measured HuR mRNA levels in hESC and 15-day EB, but only found minor differences between the two cell types (Fig. 23). In accordance with this, the HuR protein level did not change during hESC differentiation (Fig. 24, 25B). As HuR activity was reported to be regulated by its nucleo-cytoplasmic shuttling (Fan and Steitz, 1998; Kim et al., 2008a), we examined HuR cellular localization during hESC differentiation. Western blot (WB) of nuclear and cytoplasmic extracts of hESC and 15-day EB in the Shef-1 and H-181 cell lines showed that, in both cases, HuR was primarily nuclear and its location did not change during hESC differentiation. This implies that nucleo-cytoplasmic shuttling of HuR is not the main mechanism that regulates HuR activity in hESC. HuR binding to target mRNA is regulated by CHK2-dependent phosphorylation in cancer cells (Abdelmohsen et al., 2007). To determine whether HuR is also regulated by phosphorylation during stem cell differentiation, we immunoprecipitated total HuR in Shef-1 stem cells and EB, and analysed the phosphorylation status of HuR using an antibody to phosphorylated serine. Phosphorylated HuR levels did not change notably during hESC differentiation (Fig. 25A), suggesting that serine phosphorylation is not

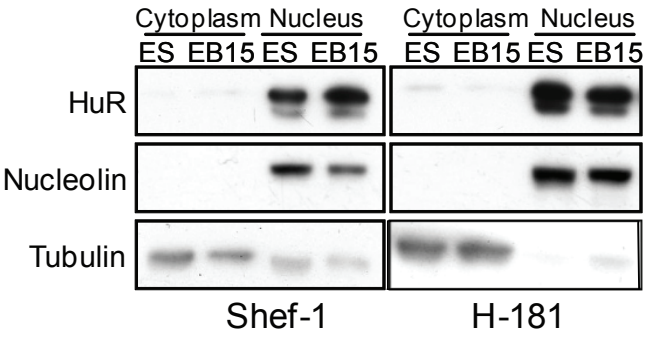


Figure 24. HuR localisation. Western blot of HuR cytoplasmic and nuclear protein fractions of Shef-1 and H-181 hESC (ES) and EB. Nucleolin and α-tubulin were used as nuclear and cytoplasmic markers, respectively.

the primary mechanism of HuR regulation during stem cell differentiation.

HuR is regulated by CARM1 (coactivator-associated arginine methyltransferase 1)-dependent methylation at Arg217 (Li et al., 2002a). To determine whether HuR is regulated by methylation during hESC differentiation, we assessed methyl-HuR levels in hESC and 15-day EB, and found a marked reduction in methylated HuR in differentiated cells (Fig. 25B).

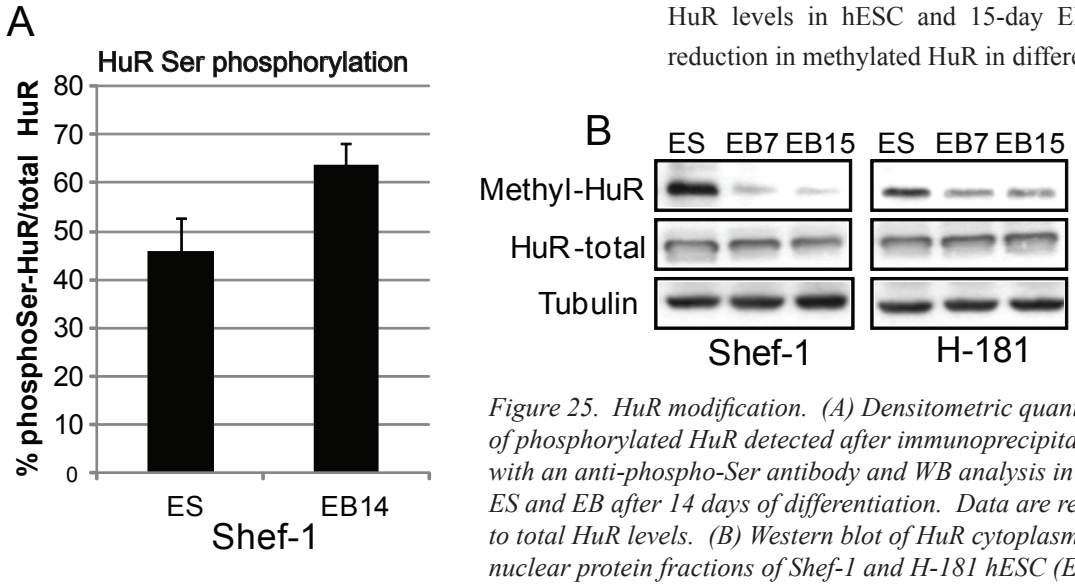


Figure 25. HuR modification. (A) Densitometric quantification of phosphorylated HuR detected after immunoprecipitation with an anti-phospho-Ser antibody and WB analysis in Shef-1 ES and EB after 14 days of differentiation. Data are relative to total HuR levels. (B) Western blot of HuR cytoplasmic and nuclear protein fractions of Shef-1 and H-181 hESC (ES) and EB. Nucleolin and α-tubulin were used as nuclear and cytoplasmic markers, respectively.

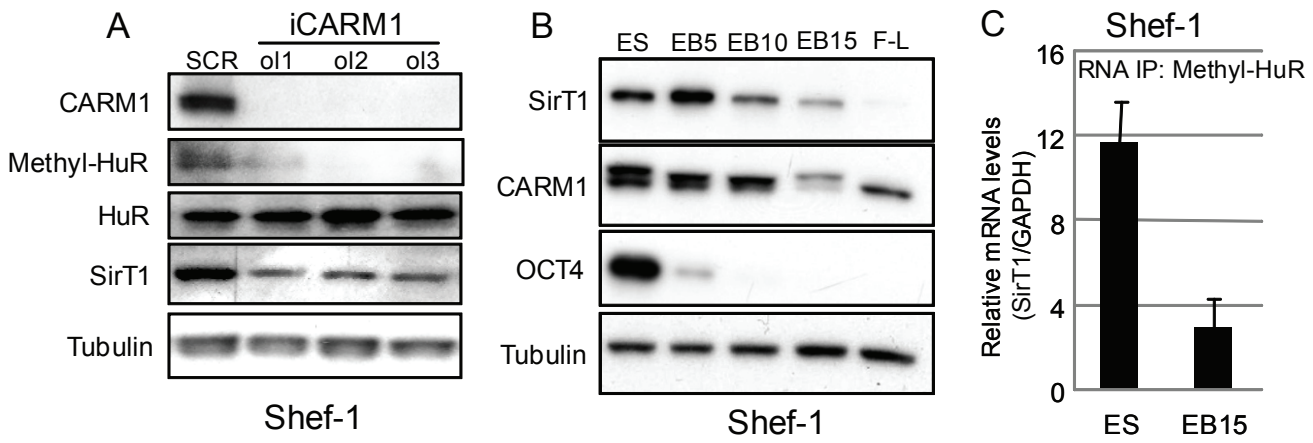


Figure 26. CARM1 methylation of HuR. (A) WB of CARM1, methyl-HuR, total HuR, SirT1 and α-tubulin in Shef-1 cells at day 3 post-transfection with control siRNA (SCR) and three specific siRNA oligonucleotides for CARM1 (iCARM1 ol1, ol2, ol3). (B) WB of CARM1 in undifferentiated Shef-1 hESC and EB differentiated for 5, 10 and 15 days (EB5, EB10, and EB15) and fibroblast-like (F-L) cells. SirT1 and Oct4 (POU5F1) were detected in the same gel. (C) RNA immunoprecipitation assay of methyl-HuR in hESC (ES) and EB differentiated for 15 days (EB15) of Shef-1 and H-181 hESC.

To determine whether this decrease depends specifically on CARM1, we used three siRNA to deplete this arginine methyltransferase in Shef-1 hESC. CARM1 knock-down resulted in the loss of methyl-HuR and in a marked decrease in SirT1 (Fig. 26A). To verify that CARM1-dependent HuR methylation regulates SirT1 mRNA stability during hESC differentiation, we measured CARM1 levels in hESC and EB over time and determined methyl-HuR binding to SirT1 during hESC differentiation. In line with our hypothesis, the decrease in SirT1 during hESC differentiation was associated with a decrease in CARM1 (Fig. 26B) and methyl-HuR binding to SirT1 was much lower in EB than in hESC (Fig. 26C).

To confirm that HuR methylation influences HuR binding to SirT1 mRNA during hESC differentiation, we compared the amount of SirT1 mRNA bound to HuR in Shef-1 cells transfected with two Arg217 HuR mutants (Arg217/Lys, Arg217/Ala) that are resistant to CARM1-dependent methylation to Shef-1 cells transfected with WT HuR. While the Arg217/Lys mutant mimics the unmethylated positive-charged wild type residue but cannot be methylated (at least by CARM1), Arg217/Ala lacks the entire side chain of the residue. We performed IP assays using an anti-V5 antibody (an epitope tag in the WT and mutant HuR constructs) and a control antibody to methyl-HuR. The HuR mutants that are resistant to CARM1 methylation bound considerably less SirT1 mRNA than did WT HuR and methyl-HuR (Fig. 27). These results suggest that SirT1 downregulation during hESC differentiation is mediated by a CARM1-dependent decrease in methyl-HuR/SirT1 mRNA binding.

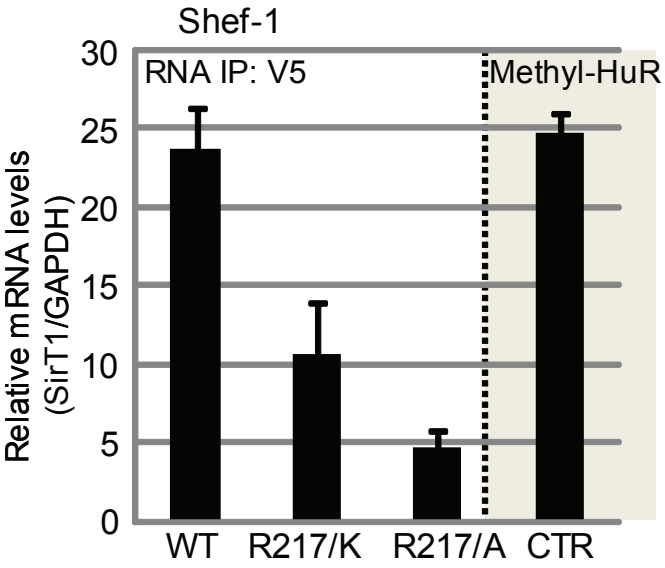


Figure 27. Mutation of HuR Arg217 impedes SirT1 mRNA binding. RNA immunoprecipitation assay with an anti-V5 antibody in Shef-1 cells transfected with pCDNA3.3 plasmid expressing WT HuR, an R217/K or an R217/A mutant HuR with V5 tag (WT, R217/K and R217/A, respectively). As control, Shef-1 cells were transfected with an empty plasmid and immunoprecipitated with an antibody to methyl-HuR (CTR) and tested in the same conditions.

As the drop in SirT1 protein levels appears more marked than that of mRNA, we studied other possible mechanisms for SirT1 protein level regulation. SirT1 translation is regulated by miR34a (Yamakuchi et al., 2008). We thus measured levels of this miRNA in our samples relative to RNU19 (Fig. 28), whose expression is considered constant in these conditions (a "housekeeping ncRNA"). This miRNA appears to be downregulated during hESC differentiation, excluding a possible role in SirT1 downregulation. Protein stability is also important in the steady-state levels of SirT1 protein, and the JNK2-dependent phosphorylation of SirT1 in the Ser27 correlates well with SirT1 stability (Ford et al., 2008). We found that phosphorylation of SirT1 at Ser27 is downregulated, as is the total protein level during hESC differentiation, but the extent of this downregulation is slightly higher than that of the total SirT1 protein decrease (Fig. 29). We thus cannot exclude that protein destabilisation due to inhibition of SirT1 phosphorylation contributes to its downregulation during hESC differentiation.

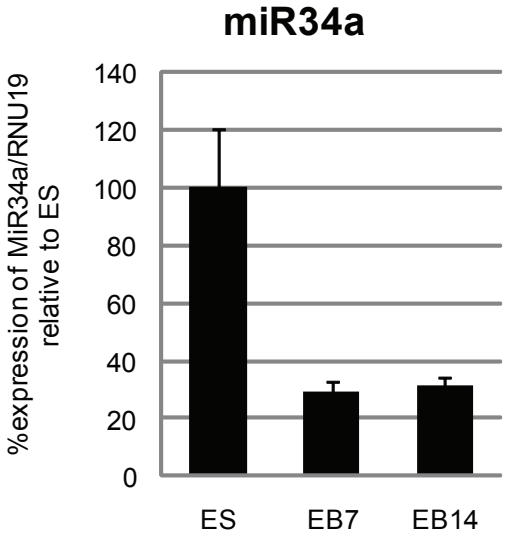


Figure 28. MiR34a expression in hESC differentiation. Time-course expression study of miR34a by q RT-PCR (relative to RNU19) in hESC Shef-1 (ES) and differentiation to EB for 7 and 14 days (EB7 and EB14, respectively).

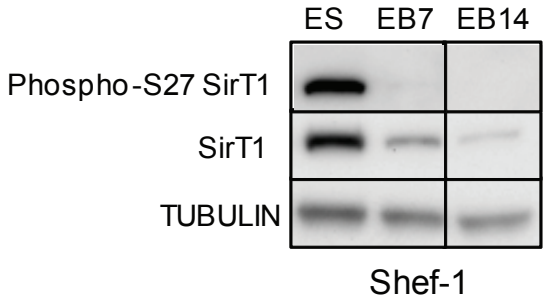


Figure 29. Phosphorylation of SirT1 Ser27. Western blot analysis of hESC Shef-1 (ES) and EB differentiated for 7 and 14 days (EB7 and EB14, respectively) with antibodies to phosphorylated-Ser27 of SirT1, total SirT1 and α-tubulin as a loading control.

SirT1 regulation of developmental gene promoters during hESC differentiation

We next investigated the role of SirT1 in hESC and the functional implication of its downregulation during hESC differentiation. SirT1 has multiple targets, including histones and transcription factors, as well as other enzymes and structural proteins. As our start point was a global hyperacetylation of histone H4 in hESC differentiation, we examined the role of SirT1 as a histone deacetylase. SirT1 was recently shown to regulate the promoter of the *MashI* gene in somatic stem cell neural differentiation in mice (Prozorovski et al., 2008); we therefore focused on the direct regulation of promoter histone acetylation during differentiation. We hypothesised that SirT1 might contribute to the negative regulation of some gene ncRNA in pluripotent hESC, and that its downregulation could contribute to the correct reactivation of these genes during differentiation.

To identify the downstream effects of SirT1 downregulation in hESC differentiation, we performed chromatin immunoprecipitation (ChIP) experiments in Shef-1 hESC using an anti-SirT1 antibody. We subsequently hybridised the immunoprecipitated DNA fragments on an Agilent human promoter ChIP-on-chip microarray containing 474,392 probes, which covered almost all described human promoters and many of the known regulatory regions. These experiments showed that SirT1 bound 428 probes (0.09% of the total), corresponding to 353 gene promoters (since some have more than one probe and some are divergent promoters that regulate two genes), four intergenic regions and four microRNA promoters; the exact genomic location of each SirT1-positive probe is shown in Table S13. Gene ontology (GO) analysis of the SirT1-bound genes showed non-random distribution, largely with respect to molecular function (Table 6, S14, S15). SirT1-bound genes in hESC are greatly enriched ($p = 10^{10} – 10^{-4}$, Table 5) for GO terms related to development and differentiation, such as developmental process, multicellular organismal development and cell differentiation (Table 6). Of the 353 SirT1-positive genes, 97 (1.76-fold enrichment; $p = 3.05 \cdot 10^{-9}$) are described by the highest-ranking GO term 0032502~developmental process

GO TERM (Biological process) (p<0.001)	Nº of	p value
GO:0032502~developmental process	97	3.05E-09
GO:0032501~multicellular organismal process	104	3.01E-08
GO:0007275~multicellular organismal development	73	1.50E-07
GO:0048856~anatomical structure development	68	2.75E-07
GO:0048731~system development	54	1.76E-05
GO:0009653~anatomical structure morphogenesis	39	3.54E-05
GO:0030154~cell differentiation	53	1.08E-04
GO:0048869~cellular developmental process	53	1.08E-04
GO:0035270~endocrine system development	6	2.45E-04
GO:0003008~system process	43	4.13E-04
GO:0007154~cell communication	94	7.75E-04
GO:0048513~organ development	38	8.78E-04

Table 6. GO terms significantly enriched in SirT1-bound genes

(Table 6). These observations suggest that SirT1 is involved in the regulation of specific developmental genes in hESC.

To validate the ChIP-on-chip data, we performed ChIP experiments in Shef-1 hESC and EB using antibodies to SirT1 and to AcK16-H4 and AcK9-H3, two known histone targets of SirT1 (Prozorovski et al., 2008; Vaquero et al., 2007b). The relative abundance of specific DNA fragments within the immunoprecipitated chromatin was assessed by quantitative PCR (q-ChIP). For this validation, we randomly selected 10 of the 97 SirT1-positive genes classified within the GO term "developmental process". These genes were delta-like 4 (DLL4), a Notch ligand required for normal embryonic vascular development; LIM homeobox 1 (LHX1), a homeodomain-containing transcription factor (TF) involved in developmental processes such as axon guidance; paired box 6 (PAX6), a TF involved in neuroectodermal definition and oculogenesis; member 6 of the wingless-type MMTV integration site family (WNT6), a secreted glycoprotein important for myocardial and neural crest development; bone morphogenetic protein 1 (BMP1), a metalloprotease that participates in embryonic patterning by cleaving matrix proteins and morphogens; Hairy/enhancer of split 7 (HES7), a TF involved in somite segmentation; T-box 3 (TBX3), a TF that affects developmental events such as sinoatrial node determination, limb bud positioning and retinal dorso-ventral patterning; serpin peptidase inhibitor, clade E member 1-plasminogen activator inhibitor type 1 (SERPINE1), a secreted protease inhibitor regulating TGFβ and EGF signalling; homeobox A5 (HOXA5), a homeobox-containing TF important in lung, intestinal and thyroid morphogenesis and blood cell differentiation; and TIMP metallopeptidase inhibitor 1 (TIMP1), a matrix protein that regulates proteases of importance in development; with GAPDH and rDNA controls. These experiments showed that all 10 genes were enriched in SirT1 in hESC (significant in most cases, $p<0.05$; Fig. 28, 29), confirming the consistency of the entire ChIP-on-chip data set. We also observed a marked increase in 15-day EB (which have very low SirT1 levels relative to hESC) for the best-known SirT1 histone targets, AcK16-H4 and AcK9-H3, in most target genes analysed (Fig. 30, 31). GAPDH and rDNA controls showed no SirT1 enrichment or significant changes in AcK16-H4 or AcK9-H3. To confirm that these genes are regulated directly by SirT1, we depleted its activity by siRNA in Shef-1 cells and used q-PCR to measure the mRNA levels of five target genes (DLL4, TBX3, SERPINE1, WNT6 and PAX6), with GAPDH as control. SirT1 knock-down resulted in significant upregulation of all five targets ($p<0.05$; Fig. 30C), supporting its role in regulating selected genes in hESC. In addition, we found that mRNA levels of eight genes were clearly upregulated in 7- and 14-day EB and in neuroectodermal cells, which are characterised by low SirT1 levels (Fig. 32). Together, these data suggest that SirT1 regulation of specific developmental

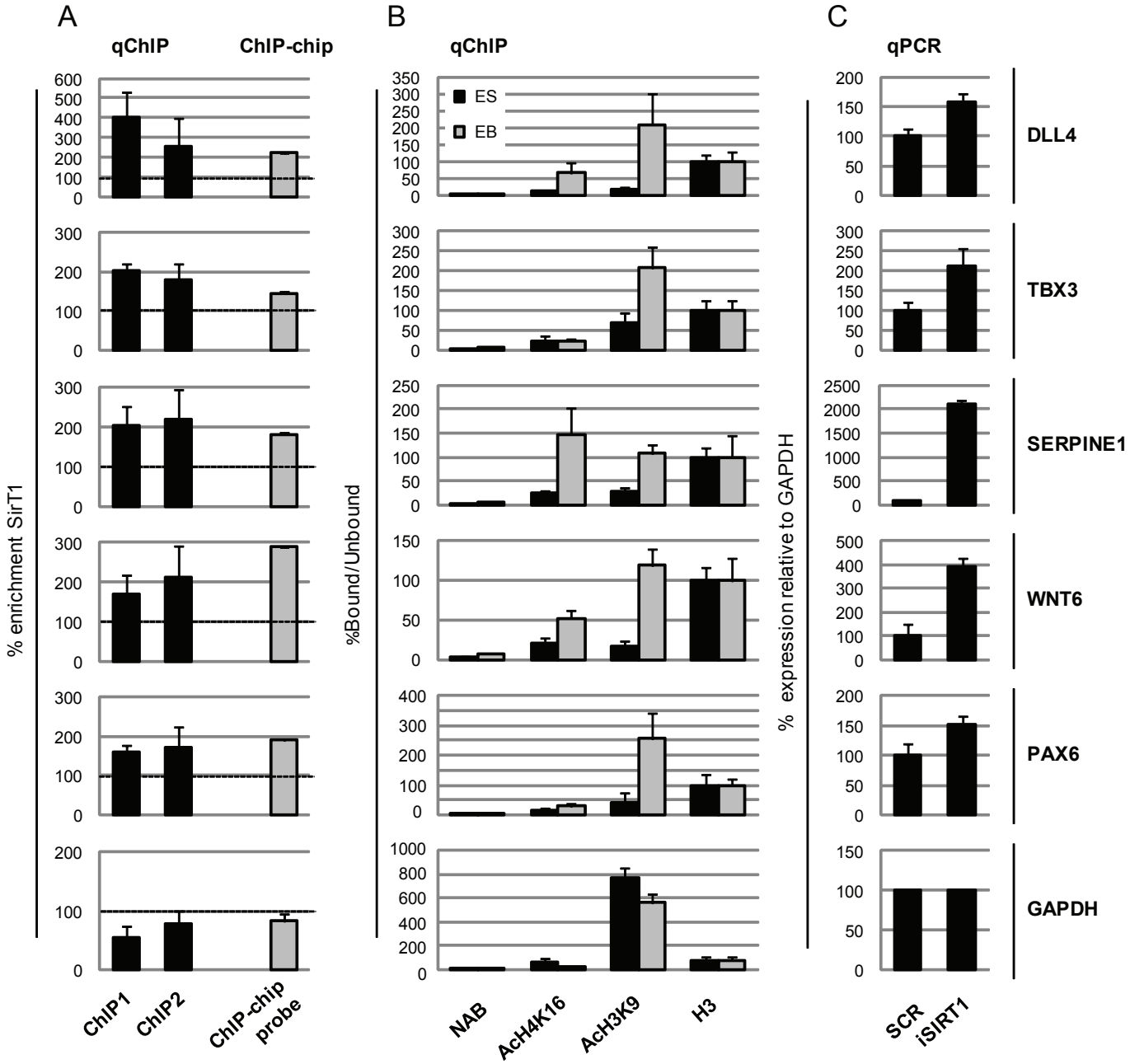


Figure 30. SirT1 binding and regulation of developmental gene promoters. (A) q-ChIP of SirT1 in Shef 1 hESC. Enrichment relative to a chromatin sample immunoprecipitated with no antibody (NAB) for the SirT1-bound regions of DLL4, TBX3, SERPINE1, WNT6 and PAX6 was studied by q-RT-PCR. Final results are expressed as the percent enrichment of the bound/unbound ratio (DNA copy number) of the SirT1 IP relative to the NAB IP in two sets of ChIP experiments (ChIP1, ChIP2). The enrichment value of the Agilent Human Promoters Array Probe (ChIP-chip probe) is also shown as the percentage of the normalised IP signal divided by the normalised input signal for the most positive probe of each gene represented (see Table S8). Primers for q-ChIP were designed around the positive probe in the ChIP-on-chip array. The GAPDH promoter was included as a negative control for SirT1 binding and histone modifications. (B) q-ChIP of acetyl-lysine 16 of histone H4 (AcH4K16) and acetyl-lysine 9 of histone H3 (AcH3K9) in Shef-1 hESC and EB cells. q-PCR corresponding to the same genomic regions described above. Final results are expressed as the bound/unbound percentage ratio (DNA copy number) for each IP and further normalised for the total H3 enrichment considered constant in chromatin. (C) Expression of these genes was measured by q-PCR in Shef-1 hESC, 3 days post-transfection with a control siRNA (SCR) and an siRNA oligo for SirT1 (iSirT1). Data are normalised with respect to GAPDH, relative to the undifferentiated hESC sample (GAPDH expression is set manually to 100%).

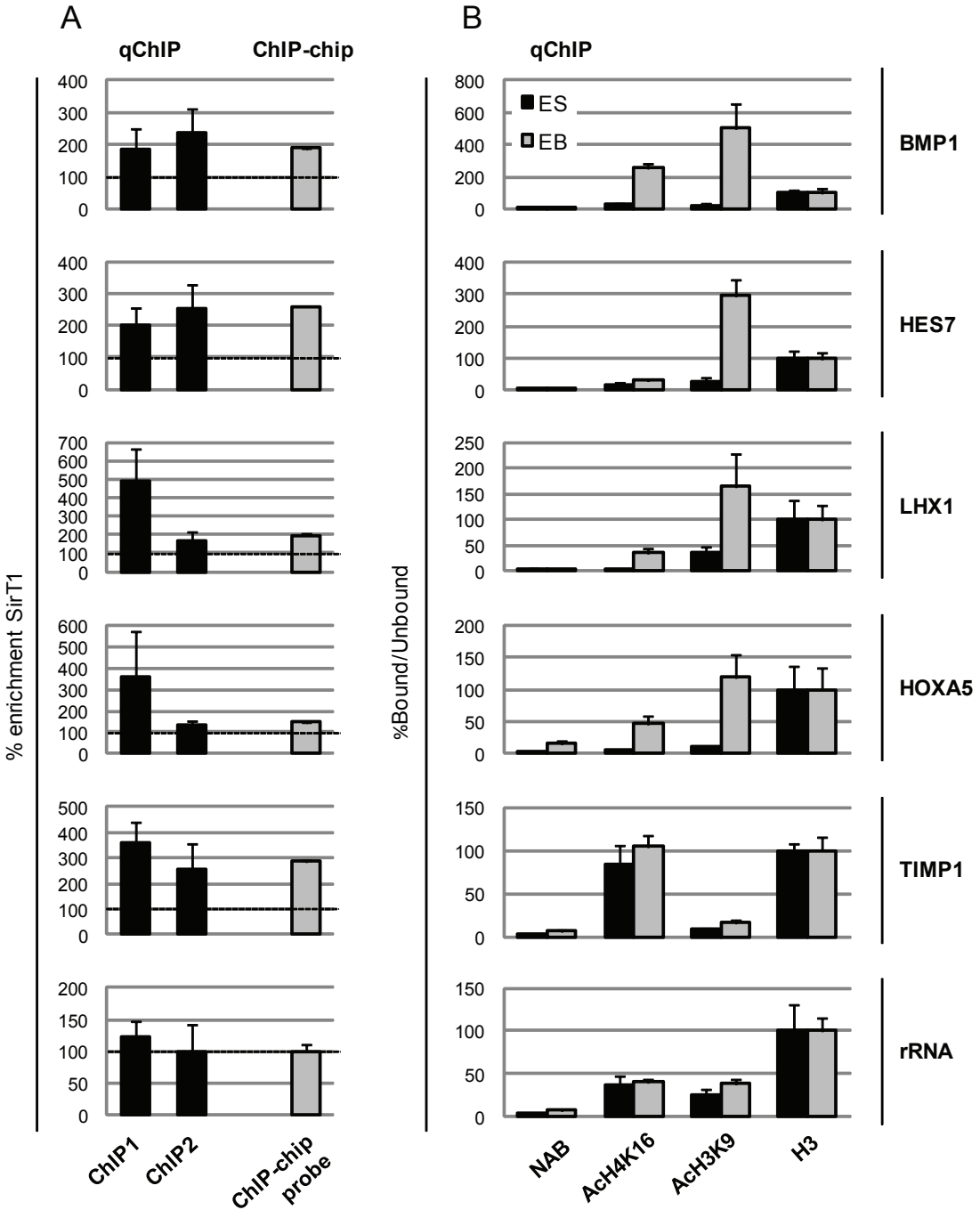


Figure 31. ChIP-on-chip validation. (A) q-ChIP of SirT1 in Shef-1 hESC. Enrichment with respect to a chromatin sample immunoprecipitated with no antibody (NAB) for the SirT1-bound regions of BMP1, HES7, LHX1, HOXA5 and TIMP1 was studied by q-RT-PCR. Final results are expressed as in Fig. 28. The promoter of the ribosomal RNA gene (rDNA) was included as a negative control for SirT1 binding and histone modifications. (B) q-ChIP of AcH4K16 and AcH3K9 in Shef-1 hESC and EB. q-PCR corresponds to the same genomic regions described above. Final results are expressed as in Fig. 30.

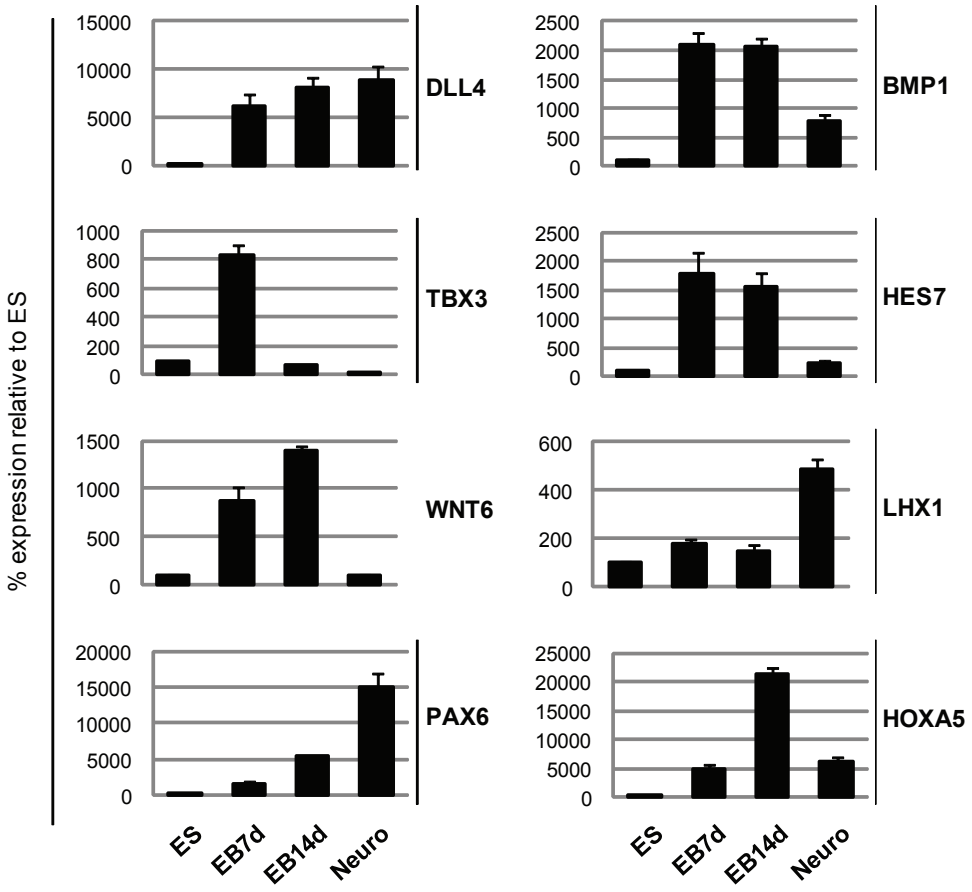


Figure 32. Expression of SirT1-bound genes during EB and neural differentiation. Gene expression was measured by q-RT-PCR in Shef-1 hESC, in differentiation to EB at 7 and 14 days (EB7 and EB14, respectively), and in the neuron-enriched sample derived from the in vitro differentiation to neuroectodermal cells. Data are normalised with respect to GAPDH, relative to the undifferentiated hESC sample (GAPDH expression is set manually to 100%).

genes during hESC differentiation is mediated by epigenetic mechanisms that involve its histone deacetylase activity.

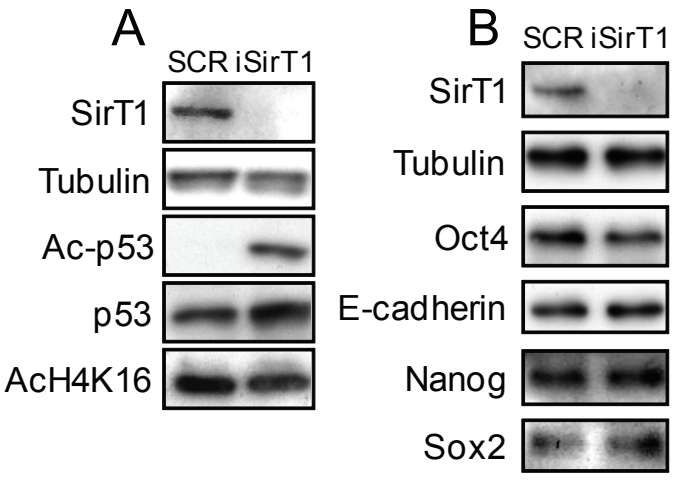


Figure 33. SirT1 knock-down in hESC. (A) Western blot of SirT1, acetyl-lysine 382 of p53, total p53, AcH4K16 and α-tubulin in Shef-1, 3 days post-transfection with a control siRNA (SCR) and SirT1-specific siRNA (iSirT1). (B) Western blot in the same samples of hESC markers Oct4, Nanog, Sox2, E cadherin.

Functional role of SirT1 in lineage specification during hESC differentiation

To study the functional role of SirT1 downregulation during hESC differentiation, we used the TaqMan Human Stem Cell Pluripotency Array and WB to compare the relative expression of stemness and differentiation markers during *in vitro* differentiation of siRNA-mediated SirT1-depleted Shef-1 cells, mouse SirT1 knockout ES cells ,and mouse ES cells genetically modified to overexpress SirT1 (Pfluger et al., 2008) with their respective wild-type controls. SirT1 downregulation by siRNA resulted in increased acetylation of the SirT1 target p53 (Fig. 31A), consistent with previously published data (Han et al., 2008). Global acetylation of H4K16, another SirT1 substrate (Vaquero et al., 2006), remained unchanged (Fig. 31A), further supporting the idea that SirT1 histone deacetylase activity is largely restricted to gene promoters (Fig. 33A). siRNA-mediated depletion of SirT1 did not notably alter the expression of the pluripotency markers OCT4, NANOG, SOX2 and E-cadherin (Fig. 33B).

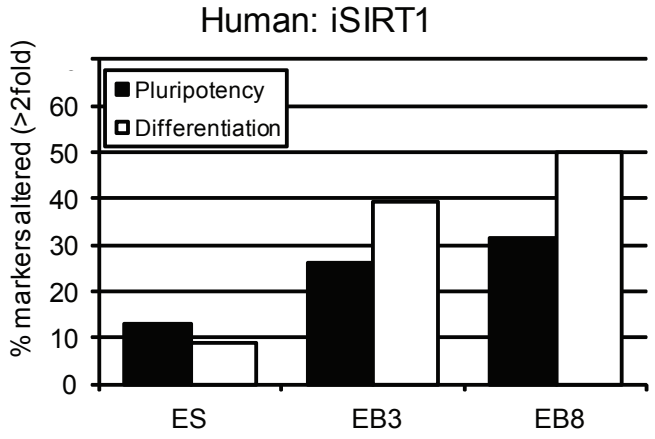


Figure 34. SirT1 knockdown in hESC differentiation. A TaqMan Human Stem Cell Pluripotency Array testing the expression of 98 genes was used to analyse Shef-1 hESC transfected with control (SCR) and SirT1 siRNA (iSirT1), at 2 days post-transfection, and after 3 (EB3) and 9 (EB9) days of differentiation of control and knocked-down samples. Results are shown as the percentage of genes catalogued as pluripotency- or differentiation-related whose expression changed by more than two-fold between control and SirT1 knocked-down samples at each differentiation stage.

Accordingly, SirT1 silencing during hESC differentiation had a greater effect on the expression of developmental markers (50% in 9-day EB) than on the expression of pluripotency markers (31% in 9-day EB) (Fig. 34). These results are further evidence that SirT1 regulates specific developmental programs during hESC differentiation.

To study the functional role of SirT1 during ES differentiation in greater detail, we used several mouse ES cell (mESC) lines in which Sirt1 was either inactivated by deletion of exon 4 containing the catalytic domain (Cheng et al., 2003), which we term Sirt1 KO, or was increased by insertion of an additional copy of the Sirt1 gene (Pfluger et al., 2008), termed Super-Sirt1.

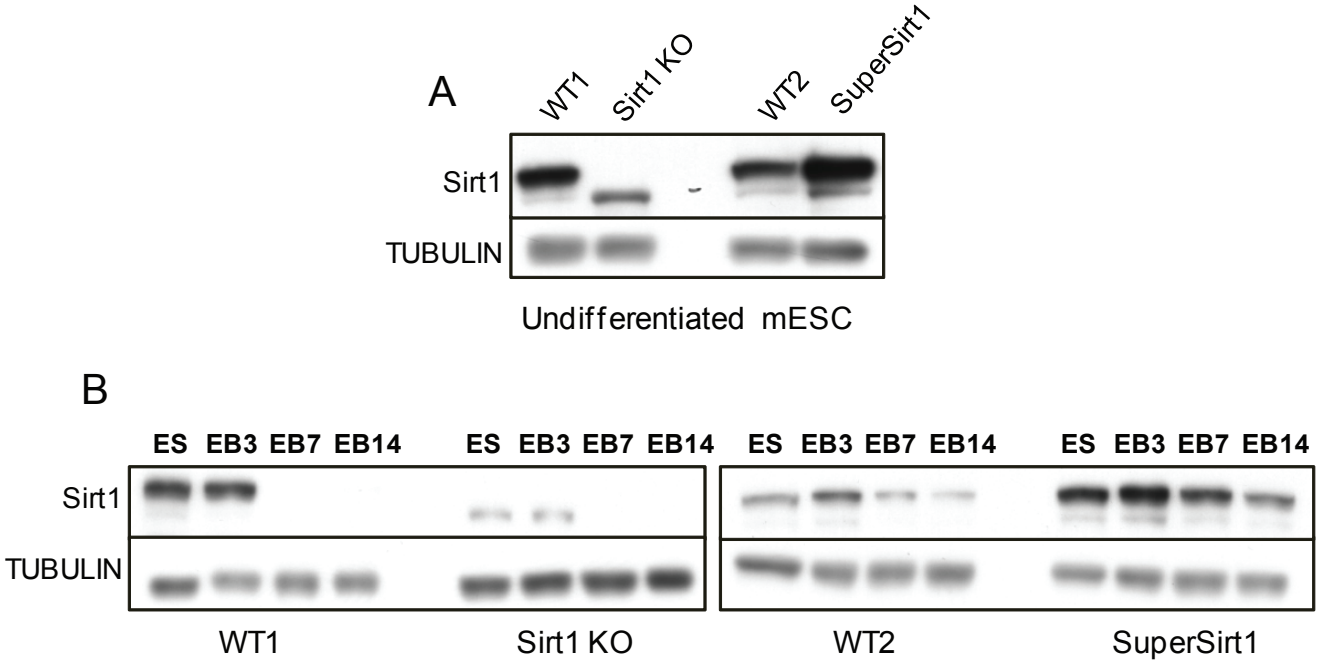


Figure 35. Sirt1 expression in mESC lines. (A) WB analysis of Sirt1 levels in mES lines: TC1 (WT1), TC1 bearing the deletion of Sirt1 exon 4 (SIRT KO), mES clone with one copy of the Sirt1 gene (see Experimental Procedures) (WT2), and mES clone bearing extra copies of the Sirt1 gene (Super-Sirt1). (B) WB of Sirt1 in the same mESC lines differentiated into EB for 3, 7 and 14 days (EB3, EB7, EB14, respectively). α-tubulin was used as a loading control.

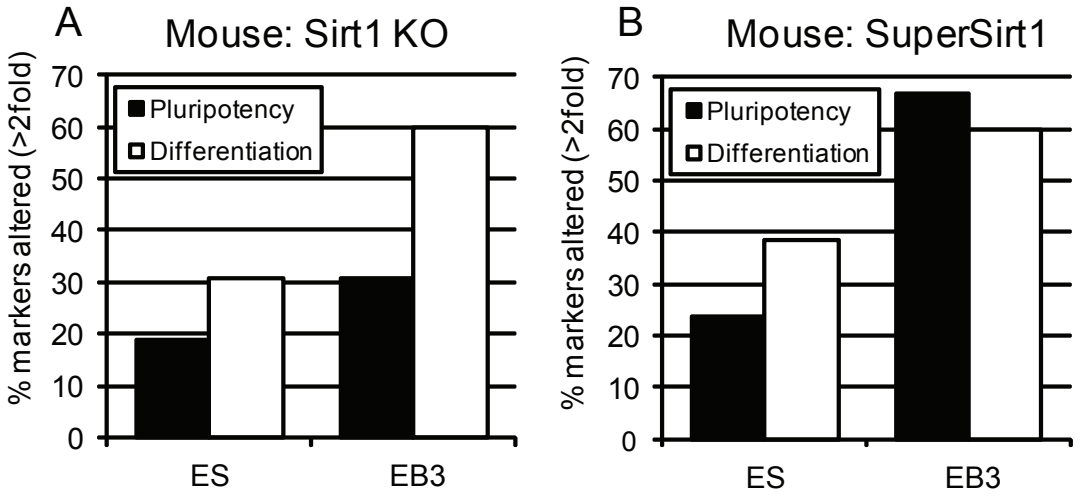


Figure 36. SirT1 modulation in mESC differentiation. (A) TaqMan Mouse Stem Cell Pluripotency Array was used to analyse TC1 mouse ES cells, the same cell line knocked-out for SirT1, and the same two lines differentiated in vitro to EB for 15 days. Results are expressed as in Fig. 31. (B) A TaqMan Mouse Stem Cell Pluripotency Array was used to study SuperSirt1 and the corresponding WT mESC, and the same two lines differentiated in vitro to EB for 15 days. Results are expressed as in Fig. 34.

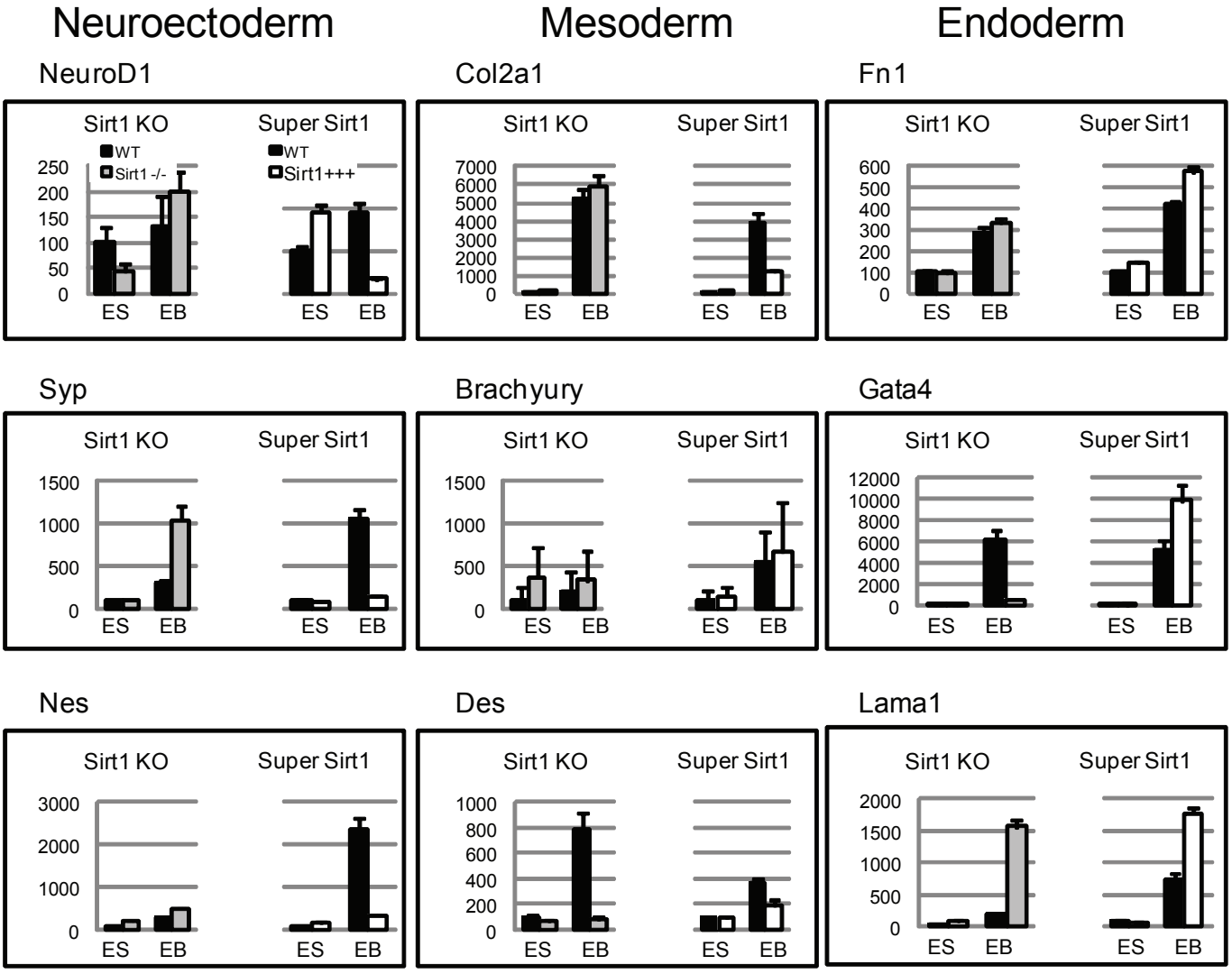


Figure 37. Expression of differentiation markers in the differentiation of Sirt1 KO (Sirt1-/-) or SuperSirt1 (Sirt1+++) mES cells. Three markers each for neuro-ectodermal (NeuroD1, Syp and Nes), mesodermal (Col2a1, Brachyury and Des) and endodermal (Fn1, Gata4 and Lama1) cells were measured by q-RT-PCR in mESC (ES) and EB at 15 days after induction of differentiation. Data (relative expression) are normalised with respect to Gapdh expression, relative to the undifferentiated ES sample for each line. Black column, WT; grey column, Sirt1 KO; white column, SuperSirt1

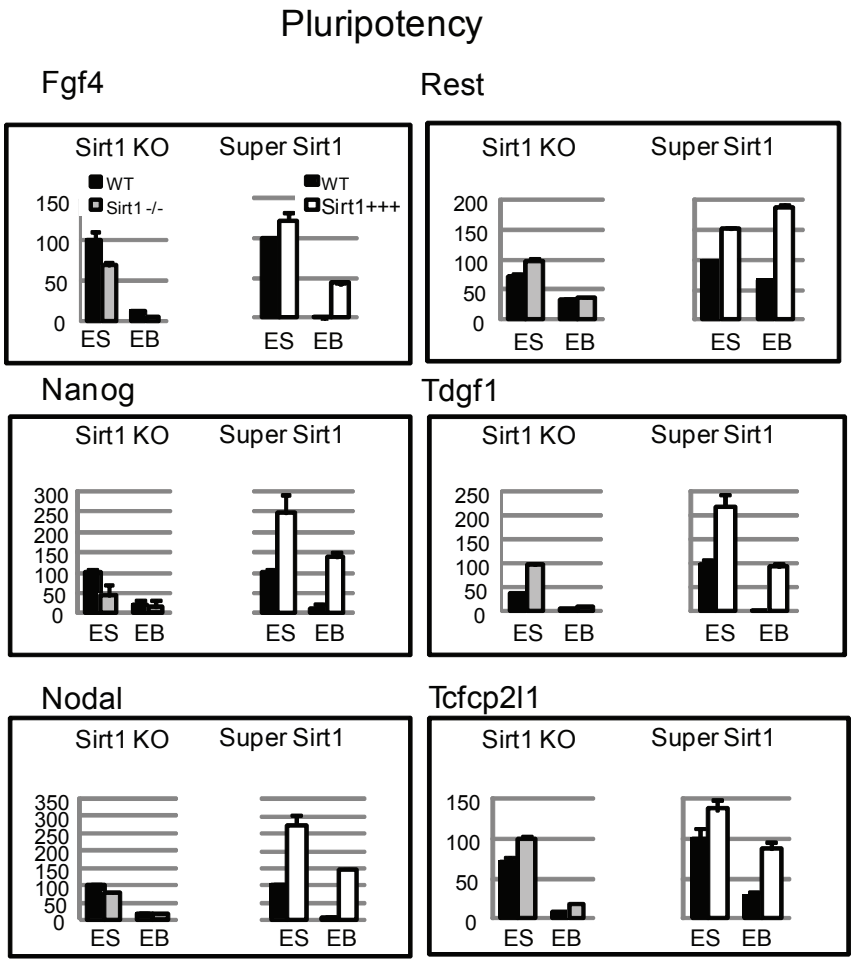


Figure 38. Expression of pluripotency markers in differentiation of Sirt1 KO (Sirt1-/-) or SuperSirt1 (Sirt1+++) mES cells. Six undifferentiated ESC markers (Fgf4, Nanog, Nodal, Rest, Tdfcp2l1 and Tdgf1) were measured by q-RT-PCR in mESC (ES) and EB at 15 days post-induction of differentiation. Data are normalised with respect to Gapdh expression, relative to the undifferentiated ES sample for each line. Black column, WT; grey column, Sirt1 KO; white column, SuperSirt1

Sirt1 expression was verified by WB in these mESC lines (Fig. 35A) and during *in vitro* differentiation (Fig. 35B). Genetic deficiency in SirT1, as well as its overexpression, had a greater effect on the expression of developmental markers than on that of pluripotency markers (Fig. 36).

We used q-RT-PCR to analyse the expression of six pluripotency markers (Fgf4, Tdgf1, Nanog, Nodal, Rest and Tdfcp2l1), three of neuro-ectoderm (NeuroD1, Syp and Nes), three of mesoderm (Col2a1, Brachyury and Des) and three of the endodermal layer (Fn1, Gata4 and Lama1) (Fig. 37, 38). Results showed that Sirt1 knockout or overexpression in undifferentiated mES cells has little impact on the expression of developmental markers (Fig. 37). As previously observed in hESC, Sirt1 KO mES cells showed no clear alteration in the expression of pluripotency markers (Fgf4, Nanog, Nodal, Rest, Tdfcp2l1 and Tdgf1), although in this case Nanog appeared to be downregulated markedly. In contrast, Super-Sirt1 mESC notably overexpressed these pluripotency markers (Fig. 38). In addition, although Sirt1 KO EB showed no clear changes in pluripotency marker expression, Super-Sirt1 EB retained notable expression of these markers, which in most cases was comparable to their expression in WT mESC. These results suggest that the functional role of Sirt1 in mouse and human ES cells might not be identical and that, in contrast to human ES cells, Sirt1 could contribute to the maintenance of pluripotency in mouse ES cells.

During differentiation of Sirt1 genetically modified mESC, we observed that both lack and overexpression of Sirt1 resulted in a marked alteration of most of the developmental markers analysed (Fig. 33), which is in accordance with our results in human ES cells. The markers that showed major alterations were those corresponding to the neuro-ectodermal layer; all three markers analysed in this layer (NeuroD1, Syp and Nes) were overexpressed in Sirt1 KO EB and notably downregulated in Super-Sirt1 EB. These findings concur with the data for human ES cells and indicate that, in humans and in mice, SirT1 might have an important role in the establishment of the neuro-ectodermal layer.

DISCUSSION

DISCUSSION

Since its birth, epigenetics has been closely connected to development and differentiation. Considering that a single cell generates a plethora of distinct specialised cells, all of which share the same genetic information, epigenetics ("above the gene") must be involved in this process. Although our knowledge of development-related epigenetic mechanisms has undergone an exponential growth in recent decades, some aspects of this field are still foggy. Moreover, as epigenetics has been implicated in the pathogenesis of various common diseases, including cancer, autoimmune disease, atherosclerosis, psychiatric disturbances and various forms of mental retardation, detailed study of epigenetic processes is now central to nearly all biomedical fields.

DNA methylation is considered a very stable epigenetic modification, because it is inherited in cell division through a well-defined mechanism and is abundant in the promoters of many stably repressed genes. In the first ontogenic steps from germ-line definition to early embryonic stages, it nevertheless undergoes radical changes. There are two waves of genome-wide demethylation-remethylation, which is even more surprising considering that there is no univocally recognised enzyme that catalyses DNA demethylation. Changes are more subtle later in the life cycle, but many genes are still regulated by DNA methylation during embryonic development, and even during adult tissue ageing. As a means of gene inactivation, methylation has been fully implicated in the process of stable silencing of undifferentiated stem cell-related genes, such as OCT4 and NANOG. Although a considerable group of genes is hypermethylated in embryonic stem cells, to date, demethylation processes in cell differentiation have been considered of secondary importance. Our principal finding in this regard is that expression of certain genes is regulated through DNA demethylation during hESC differentiation. These genes share common specific functions, particularly in late differentiation processes of mesodermal-immune system specification. From an evolutiionary perspective, this indicates that proteins with this specific function could have evolved to be regulated through DNA methylation in differentiation.

Another process in which genomic methylation is profoundly altered is cancer. Aberrant DNA methylation in cancer has its own features, consisting of a loss of global methylation and hypermethylation of gene promoters, especially in genes whose inactivation offers an advantage for cancer progression, commonly termed tumour suppressor genes (TSG) (Feinberg and Tycko, 2004; Jones and Baylin, 2007). Genes undergoing such alterations in cancer are reported to be repressed in hESC by the establishment an ES-specific mark, bivalent chromatin domains consisting of activating (H3K27 trimethylation) and repressing (H3K4 trimethylation) histone marks that keep them poised for activation while predisposing them to aberrant promoter hypermethylation in adult cancers (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007) (Fig. 39). Here we attempted to analyse another level of complexity, whereby some genes that are often aberrantly hypermethylated

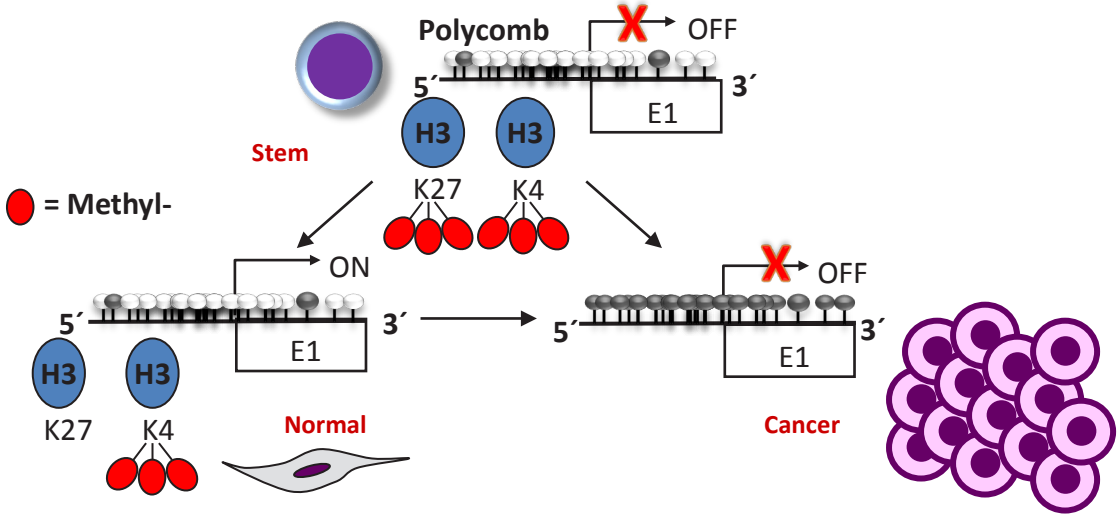


Figure 39. Classical model for bivalent domain-mediated promoter hypermethylation in cancer. TSG promoters are frequently marked in hESC with the bivalent domain and are unmethylated. Following differentiation, these genes are normally activated in the differentiated cell. During tumour transformation, these promoters are aberrantly methylated at CpG, leading to gene silencing (based on (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007).

in cancer are also frequently hypermethylated in hESC.

Based on promoter methylation status in hESC, we established two categories of cancer hypermethylated genes: Class A genes, which are frequently unmethylated in hESC, and Class B genes, which are frequently hypermethylated in hESC. As we unexpectedly found that a substantial proportion of genes in both groups were also frequently hypermethylated in normal differentiated tissues, we established two new subcategories of cancer hypermethylated genes: subcategory I for genes that are generally unmethylated in normal tissue, and subcategory II for genes that are generally hypermethylated in normal tissue. The biological interpretation of aberrant methylation within Class A and B TSG and their two subcategories is completely different. Class A-I genes are frequently hypermethylated in cancer but not in normal tissues or hESC. These genes are assumed not to be regulated by DNA methylation during normal development, and their hypermethylation in cancer would thus always be interpreted as aberrant. Class A-II genes are frequently methylated in CCL and sometimes in normal tissues, but rarely in hESC. Methylation of these genes might be important for lineage specification, and should be considered aberrant only when found in a tumour type in whose corresponding normal tissue it is not hypermethylated. Class B-I genes (excluding *ASCL2*, *NPY*, and *SLC5A8* genes, whose promoter DNA hypermethylation in hESC lines could be due to the culture process) are frequently hypermethylated in hESC and cancer cell lines, but never in normal tissues; this suggests that loss of methylation at the promoters of these genes might be important in the loss of pluripotency during development. Hypermethylation of Class B-I genes in cancer should always be considered aberrant. Class B-II genes are frequently hypermethylated in hESC and in cancer cells but, as they are also sometimes methylated in normal tissues, their hypermethylation in cancer should only be considered aberrant in tumour types in whose normal tissue counterparts they are completely unmethylated. The fact that not all genes frequently hypermethylated in cancer are completely unmethylated in all normal tissues is a very important finding in cancer epigenetics (Feinberg and Tycko, 2004), because promoter hypermethylation of a gene in a specific tumour type should not be considered aberrant when it is hypermethylated in the normal counterpart.

Comparison of our DNA methylation results with previous data on the histone modification profile and Polycomb occupancy of these genes in embryonic stem cells (Lee et al., 2006a; Mikkelsen et al., 2007; Zhao et al., 2007) showed that only around one-third of the genes frequently hypermethylated in cancer presented the bivalent chromatin domain (trimethyl-K4/K27H3) in hESC. This indicates that, at least among genes tested here, promoter occupancy with PRC and the bivalent domain in stem cells can be considered predictive of and predisposing to cancer promoter hypermethylation for less than half of the genes. Within our four gene categories, Class A genes were more enriched in Polycomb and bivalent marks than Class B genes. This suggests that a scenario involving bivalent chromatin domains and Polycomb occupancy of cancer methylated genes in embryonic stem cells (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007) could be more frequent in Class A than in Class B genes. The Class II genes, which we suggest are involved in lineage specification since their promoter methylation is tissue-dependent, were much less frequently occupied by Polycomb proteins and showed fewer bivalent marks than Class I genes, which we implicate in early differentiation. Lower levels of the bivalent mark in Class II genes were due primarily to low levels in Class BII genes, implying that Class BII is relevant to lineage specification. Zhao *et al.* (Zhao et al., 2007) proposed that genes harbouring bivalent marks are involved primarily in early differentiation, whilst those that lack both are involved in lineage specification. We found that Class I genes are enriched in the bivalent mark and Class B-II genes are frequently depleted of both marks. Our gene ontology classification of all four classes of genes associated Class I genes with biological processes involved in early differentiation, and most Class B-II genes with lineage specification processes (Table S16), coinciding with the data from Zhao *et al.*

Analysis of the DNA methylation and expression status of four of the genes identified in the methylation arrays (MGMT and SLC5A8 (Esteller et al., 2000; Li et al., 2003) from Class B-I, and PYCARD and RUNX3 (Conway et al., 2000; Li et al., 2002c) from Class B-II) showed that promoter hypermethylation was always associated with gene repression, but its absence in somatic primary tissues did not necessarily involve gene upregulation. This was the case for SLC5A8, in which there was no overexpression following promoter demethylation in peripheral lymphocytes. We therefore hypothesised that promoter hypermethylation of the Class B cancer methylated genes in hESC (excluding those whose promoter DNA hypermethylation depends on prolonged *in vitro* culture) can be a natural process used by stem cells to ensure silencing of genes whose expression is associated with stemness. When stem cells differentiate, these genes can lose their promoter

hypermethylation, which would allow gene expression. This does not imply, however, that the gene has necessarily been activated; this is shown by the fact that some of these genes remain repressed in differentiated tissues. Loss of promoter methylation during differentiation could simply maintain these genes poised for activation until they are later required by the somatic cell. The lack of hypomethylation-associated activation in some mature tissues suggests that signals other than the absence of methylation are necessary for activation of these genes.

By forcing the *in vitro* differentiation of the hESC line Shef-1, we identified 12 and 25 genes that are demethylated during neuron and F-L differentiation, respectively. Three of these genes are common to both groups, suggesting that they might be involved in early differentiation processes, further supported by the fact that two of the genes were Class B-I, which we found to be involved in early development. Whilst the majority of genes demethylated during F-L differentiation were Class B-II, none of those demethylated during neuron differentiation belonged to this category. This could be explained by the finding that 10 genes, which were demethylated during neuron differentiation and belonged to neither Class I nor II, were not classified as "unmethylated in at least one normal tissue type analysed" as their intermediate methylation levels in normal tissues did not allow classification into any of the four categories. Moreover, there were many more hypomethylated genes (relative to Shef-1) in normal brain than in neural Shef-1-derived cells. These observations suggest that i) *in vitro* hESC differentiation does not reproduce all epigenetic features of *in vivo* differentiation and ii) the unguided F-L differentiation of our hESC achieves more epigenetic hits *in vivo* than does neural differentiation.

When we extended the analysis of F-L differentiation-associated gene demethylation to a larger gene set comprising most of the genome, we observed that the proportion of demethylated genes is maintained. More than 200 genes were specifically demethylated in this lineage, mainly associated to specific, late mesodermal differentiation. Most were implicated in immune system function and immune cell differentiation or in cell signalling, and were localised in membranes or secreted. This is interesting because F-L cells have mesodermal differentiation markers, but are not specifically directed towards the haematopoietic lineage. We therefore hypothesise that expression of these genes is unlocked earlier in mesodermal differentiation and is later activated through other mechanisms at specific stages in immune cell development. This could have important implications, as it has long been known that many alterations in DNA methylation are linked to immune cell diseases, due both to uncontrolled proliferation, as is the case of leukaemia and lymphomas, and to uncontrolled activation in the case of autoimmune diseases (Javierre et al., 2008). For example, the ICF syndrome is a rare genetic condition characterised by severe congenital immunodeficiency that leads to death before adulthood, due to mutation of the DNA methyltransferase DNMT3B (Ehrlich et al., 2008).

The observation that genes like *DLC1* are demethylated and overexpressed specifically during F-L, but not neural differentiation of Shef-1 cells suggests that loss of DNA methylation-dependent expression of this gene is involved in lineage specification. This is supported by the essential role of *DLC1* in embryonic development, whereby *DLC1*-deficient mice are non-viable (Durkin et al., 2005).

Finally, we wondered whether the methylation-dependent repression of cancer genes in hESC is a molecular process associated with embryonic development or if, in contrast, it is an epigenetic mechanism involved in the maintenance of stemness. $CD34^+$ somatic stem cell progenitors featured numerous genes frequently hypermethylated in cancer that are repressed by promoter hypermethylation. This suggests that, at least for these genes, the process could be associated with stemness status regardless of the ontogenetic stage of the cell, rather than being an event restricted to embryonic development. Since $CD34^+$ cells are primary non-cultured cells, we can also discount the possibility that *in vitro* culture of the hESC is responsible for hypermethylation, in accordance with previous findings (Bibikova et al., 2006; Rugg-Gunn et al., 2005; Rugg-Gunn et al., 2007).

By comparing the DNA methylation status of $CD34^+$ progenitor cells with those of two types of primary cells that are terminally differentiated from the former (PBL and neutrophils), we identified several genes that lost methylation specifically in just one lineage. In conjunction with the fact that most sequences identified were sometimes hypermethylated in NPT and most were previously classified as Class B-II genes, this suggests that genes hypermethylated in $CD34^+$ progenitor cells that are demethylated during differentiation are those primarily involved in lineage specification. That none of the sequences identified in the $CD34^+$ progenitor cells was of Class B-I might well be because $CD34^+$ cells are not the primary hematopoietic progenitors and because Class B-I genes lose methylation in

the transition from earlier progenitor stem cells to CD34+ cells. This explanation is consistent with the putative role of Class B-I genes in early development (Zhao et al., 2007), but requires further study.

From our point of view, the finding that some cancer methylated genes are also frequently hypermethylated in adult stem cells is particularly important to our understanding of aberrant methylation in cancer. This phenomenon could be relevant in a specific aspect of cancer biology. In recent years, a new theory supported by several findings postulates the existence of a stem cell subpopulation in the cancer cell population; these stem cells would be responsible for tumour generation and maintenance (Polyak and Hahn, 2006; Vermeulen et al., 2008). The tumour mass would be generated by a small population of cancer stem cells, which exclusively retain the ability for self-renewal and tumorigenicity. This subpopulation has been defined for several tumour types by specific markers (CD133 for brain, colon, lung, liver, pancreas and prostate cancer, CD44 for colon, breast, pancreas, prostate, head and neck tumours, and $CD34^{+}/CD38^{-}$ for haematological malignancies (Vermeulen et al., 2008)). The tumourigenicity of these cells is assayed by their ability to give rise to neoplasias at very low cell titres in NOD/SCID mice, whereas the total cancer cell population depleted of these cells is unable to generate a tumour in this model. These cancer stem cells (CSC) appear to share many characteristics with other stem cell types, such as self-renewal, asymmetric division giving rise to more differentiated progenitors, and a slow replication rate that could decrease sensitivity to current cancer treatments. The definition of this subpopulation is highly controversial, as it is difficult to fully define a very small, peculiar population in the sea of a heterogeneous tumour mass, based on an *in vivo* assay in immunodeficient mice. For example, the degree of immunodeficiency in the mouse model can greatly alter the result of the serial dilution tumour xenograft tests, thereby altering the definition of the CSC population (Quintana et al., 2008).

We found that a group of TSG is hypermethylated in hESC, and that many of are still methylated in adult stem cells, as observed in the CD34+ population. This raises the question as to whether these genes are in fact aberrantly hypermethylated in cancer, or if cancer derives from a cell subpopulation that was unable to be efficiently demethylated at these promoters, which would activate these TSG/differentiation genes. This population would possibly behave as a cancer stem cell intermediate, sharing features of both stem cells and cancer cells. In the context of the hypothesis of the stem cell origin of

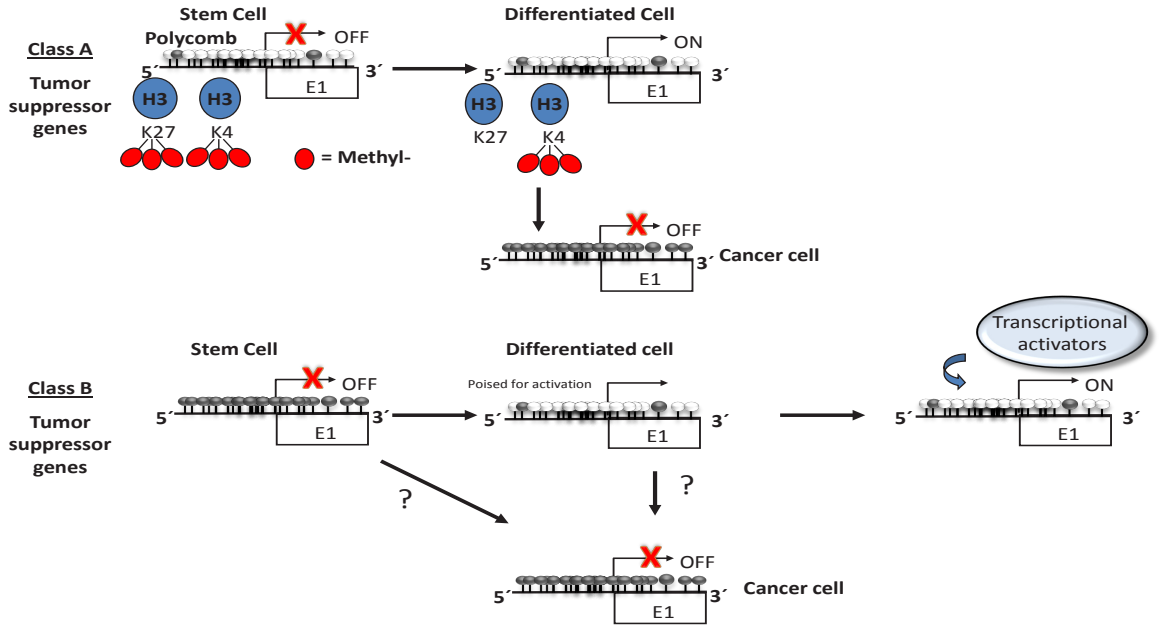


Figure 40. Model for promoter hypermethylation in cancer. Model for a Class A TSG (top). These promoters are inactivated in stem cells by the presence of the bivalent mark and are unmethylated. Following differentiation, these genes are normally activated in the differentiated cell. During tumour transformation, these promoters are aberrantly methylated at CpG, leading to gene silencing. Model for a Class B TSG (bottom). These genes do not usually have the bivalent mark in hESC, but are inactivated by promoter hypermethylation. Methylation is reversed during differentiation in tissues that potentially express these genes, although additional histone modification-based mechanisms are likely to be necessary for their full activation. In cancer, these genes are again hypermethylated. Cancer hypermethylation might be the result of aberrant remethylation of the promoter (as for class A genes) or might represent a remnant of hESC methylation, maintained by some cells that have not achieved the appropriate demethylated state during differentiation, and thereby have an advantage in the first steps of cancerogenesis.

cancer, the hypermethylation of some Class B genes in adult stem cells suggests that their aberrant methylation in cancer can be understood as a defect in establishing an unmethylated promoter during differentiation, rather than as an anomalous process of *de novo* hypermethylation (Fig. 40).

Using this approach, we identified two genes, *AIM2* and *RUNX3*, that were hypermethylated and repressed in $CD34^+$ haematopoietic progenitor cells and that were demethylated and expressed in myeloid and lymphoid lineages, respectively. Both genes are aberrantly hypermethylated in cancer (Li et al., 2002c; Woerner et al., 2007), which indicates that genes frequently hypermethylated in cancer can be naturally repressed by promoter methylation not only in hESC, but also in somatic stem cells. Moreover, the lineage-specific loss of methylation and upregulation of these two genes suggests that their expression is important in lineage specification during haematopoietic differentiation and, more importantly, that this process can be regulated by DNA methylation. RUNX3 is a well-known transcription factor that regulates lineage-specific gene expression in developmental processes (Levanon et al., 2003). Our observation that *RUNX3* loses methylation and is expressed during lymphoid, but not myeloid development is consistent with studies showing the need for RUNX3 in T cell development during thymopoiesis (Egawa et al., 2007; Woolf et al., 2003), and that *RUNX3* knockout mice have a defined T cell phenotype (Levanon et al., 2002; Li et al., 2002c). Finally, considering the available evidence and following similar reasoning, the aberrant hypermethylation of *RUNX3* in the Raji human lymphoid CCL could be understood as a failure of $CD34^+$ cells to lose the promoter methylation necessary to reactivate the gene during haematopoietic differentiation.

In addition to DNA methylation, histone modification is a potential mechanism for epigenetic gene regulation during hESC differentiation. The second part of this study addressed histone modification-based mechanisms in stem differentiation. Some of these mechanisms have been identified as central to hESC pluripotency and differentiation; these processes are based principally on the interplay of a web of key transcription factors and chromatin-modifying enzymes that act mainly through histone methylation. Histone acetylation has been analysed in some studies of hESC differentiation, but no compelling role has yet been identified. Golob *et al*. (Golob et al., 2008) reported that global acetylation of H3K9 increases during mESC and hESC F-L differentiation, while Krejci *et al*. (Krejci et al., 2009) showed that this same residue loses acetylation during retinoic acid-induced endoderm-like differentiation of hESC. Other research pointed to a role for HAT and HDAC complexes in pluripotency maintenance and ESC differentiation. For example, Baltus *et al*. (Baltus et al., 2009) showed a positive role for mSin3A-HDAC complex in mESC pluripotency maintenance; Zhong *et al*. (Zhong and Jin, 2009) demonstrated that HAT p300 deletion, although it did not affect self-renewal capacity when mESC were maintained in undifferentiated conditions, caused an abnormal expression pattern of germ layer markers when differentiation was induced by EB formation. Recent evidence from mouse models indicates that SirT1 is a central regulator of embryonic (Han et al., 2008) and somatic (Prozorovski et al., 2008) stem cell function. Our results extend these observations by showing that SirT1 might also be an important regulator of hESC differentiation, and suggest a possible molecular pathway for SirT1 regulation in stem cells. Indeed, we show that SirT1 downregulation during hESC differentiation ultimately depends on CARM1-dependent HuR Arg217 methylation. It was recently suggested that Carm1 helps to maintain pluripotency in mESC through regulation of histone methylation at the promoters of specific pluripotency genes (Torres-Padilla et al., 2007; Wu et al., 2009). In addition, Wu *et al.* showed that developmental factors are overexpressed by iCarm1, although they did not address how these genes are upregulated. As there is an overlap between the genes overexpressed after Carm1 interference in mESC with those whose promoters are bound by SirT1 in hESC (Table S17). We consider that our data and those of Wu *et al*. help to explain the central role of CARM1 in pluripotency. CARM1 downregulation can have a direct effect on pluripotency by regulating chromatin structure, and an indirect effect on priming developmental genes through SirT1 (Fig. 41).

During differentiation, the decrease in CARM1 is associated with a decrease in HuR methylation and, consequently, of HuR/SirT1 binding. This results in less SirT1 mRNA and protein, as well as the epigenetic reactivation of its target developmental genes. This hypothesis would explain our observation that, at difference from iCARM1, iSirT1 alone cannot induce hESC differentiation. CARM1 acts on both pluripotency and differentiation genes, and its knock-down is thus able to force differentiation. Repression of pluripotency genes is absolutely necessary to induce ES cell differentiation and, in hESC, SirT1 acts only on developmental genes.

As the principal mechanism thought to regulate HuR function is a change in its cytoplasmic abundance (Kim et al.,

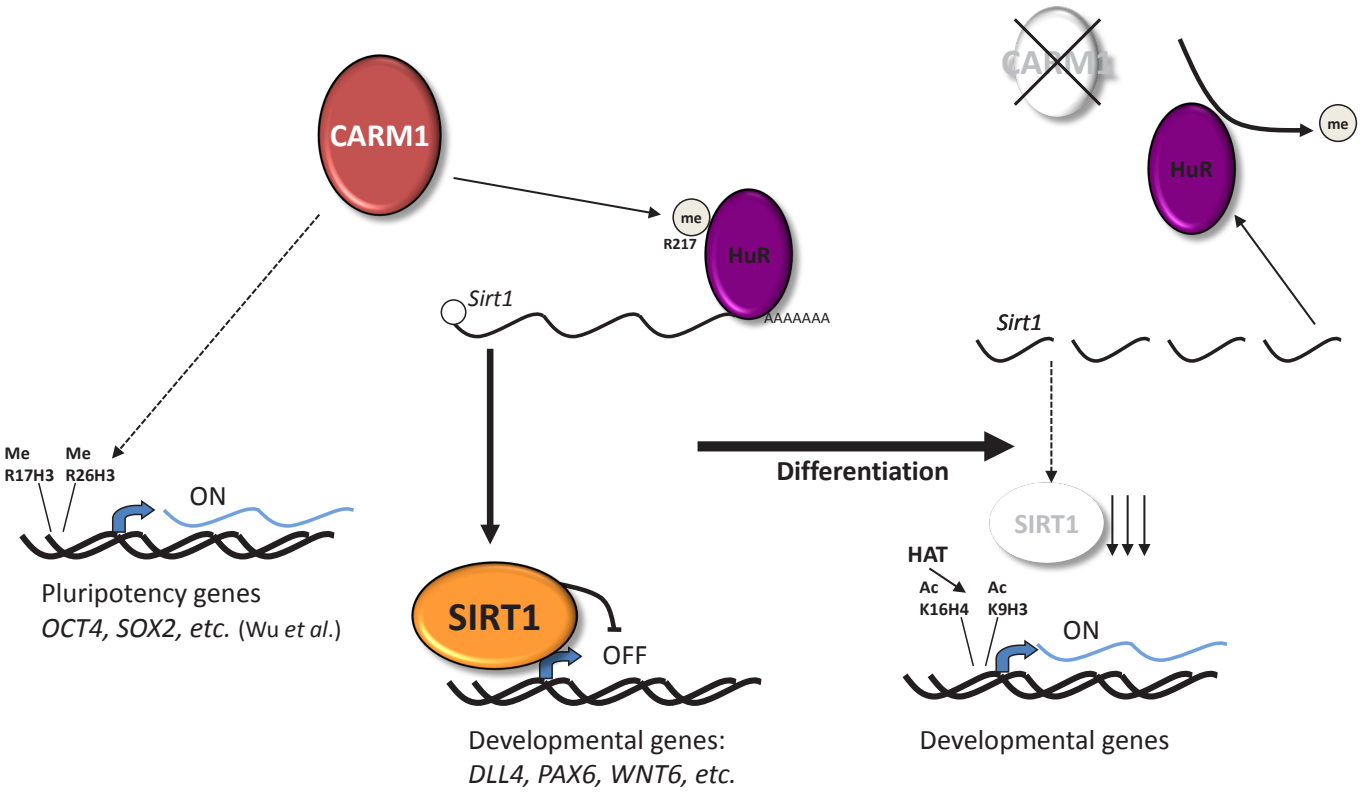


Figure 41. Model for SirT1 action on developmental gene promoters during hESC differentiation. As described by Wu et al. (Wu et al., 2009), Carm1 regulates pluripotency gene promoters by histone methylation in mouse ES cells. In pluripotent hESC, CARM1 methylation of HuR increases HuR/SirT1 binding and, consequently, SirT1 mRNA stability and the SirT1 protein level. In these conditions, SirT1 binds to the promoter and epigenetically represses specific developmental genes such as DLL4, PAX6 and WNT6.

2008a), we would have predicted this as the cause of reduced association between HuR and the SirT1 transcript. Our results nonetheless suggest that HuR/SirT1 mRNA binding relies on CARM1-dependent HuR methylation; this is consistent with a report showing the ability of CARM1 to methylate HuR and increased HuR/RNA binding activity mediated by HuR methylation at Arg217 (Li et al., 2002a). HuR regulation of SirT1 mRNA is not a new finding; HuR was recently found to regulate SirT1 in cancer cells (Abdelmohsen et al., 2007). Here we demonstrate that HuR also regulates SirT1 during hESC differentiation; in conjunction with the reported role of HuR in myogenic differentiation (Figueroa et al., 2003), this suggests that HuR regulation of SirT1 could be a shared mechanism of stem cell differentiation.

Our data indicate that Sirt1 might help to maintain pluripotency in mouse but not in human ESC. In mouse, we found that several pluripotency markers, including Fgf4, Nanog, Nodal, Tdgf1, Rest and Tdfcp2l1, are overexpressed in Super-Sirt1; some of these markers are downregulated in Sirt1 KO mESC. In addition, although Sirt1 KO embryonic bodies showed virtually no changes in pluripotency marker expression, Super-Sirt1 EB retained notable expression of these markers that, in most cases, was comparable to that in WT ES cells. These data indicate that Sirt1 might contribute to pluripotency maintenance in mESC. Even if Sirt1 would help to maintain pluripotency in mESC, it is not strictly necessary, as Sirt1 KO mESC do not differentiate spontaneously, Super-Sirt1 mES can differentiate into EB, and both Sirt1 KO and Super-Sirt1 mice are viable (Cheng et al., 2003; McBurney et al., 2003b; Pfluger et al., 2008). In man, however, SirT1 does not appear to have a clear role in hESC pluripotency, as iSirt1 in Shef-1 cells does not in itself induce differentiation. Moreover, iSirT1 does not result in downregulation of pluripotency markers, which could explain the lack of phenotypic differentiation after SirT1 downregulation. In contrast to mice, therefore, SirT1 downregulation in hESC does not appear to affect expression of pluripotency markers; this might indicate that the role of SirT1 role in ES cells in this regard is not identical in mouse and human cells.

Our results show that SirT1 downregulation is necessary to establish correct, specific differentiation programs during human and mouse ESC differentiation for two reasons: i) SirT1 binds to and epigenetically regulates specific developmental genes in pluripotent hESC, and ii) expression of markers of

pluripotency and, above all, of differentiation is clearly altered in differentiating SirT1-knocked-down hESC and Sirt1 KO/Super-Sirt1 mESC. We show that some downstream effects of SirT1 downregulation are mediated by the epigenetic reactivation of specific developmental genes, which is consistent with the role of Sirt1 in cell differentiation as a component of the Polycomb repressive complex 4 (PRC4) (Kuzmichev et al., 2005). The role of SirT1 in hESC function must nonetheless be more complex, as SirT1 downregulation is also associated with p53 hyperacetylation (this study and (Cheng et al., 2003; Han et al., 2008)). Of the ten SirT1 target genes we selected for ChIP validation, three (*LHX1*, *PAX6* and *WNT6*) have been associated with neural development (Kania et al., 2000; Schmidt et al., 2007; Wawersik and Maas, 2000) and another three (*DLL4*, *TBX3* and *PAX6*) are reported to have a role in retinal morphogenesis (Lobov et al., 2007; Wawersik and Maas, 2000). This suggests that SirT1 contributes to neural fate determination and retinal formation during embryonic development. This possibility is supported by the following arguments: i) expression of the neuro-ectodermal markers NeuroD1, Syp and Nes was clearly upregulated during differentiation of mESC lacking Sirt1 and strongly downregulated during differentiation of Sirt1-overexpressing mESC, ii) SirT1 involvement in determining the fate of neural progenitors, and iii) the notable developmental defects of the retina in SirT1-deficient mice (Cheng et al., 2003; McBurney et al., 2003b).

Based on evidence from our work, a previously undescribed epigenetic pathway appears to be involved in hESC differentiation, in which SirT1 regulates differentiation in a HuR-CARM1-dependent fashion (Fig. 40). This pathway involves the epigenetic regulation of key developmental genes such as the neuro-retinal morphogenesis effectors *DLL4*, *TBX3* and *PAX6*. In conjunction with the phenotype shown by different strains of Sirt1-deficient and Super-Sirt1 mice, our results indicate that whereas SirT1 has a minor role in promoting or impairing hESC differentiation, it contributes to the establishment of specific developmental/differentiation programmes of particular relevance for neuro-ectodermal fates.

The SirT1-mediated modulation of stemness and developmental genes might not be merely an additional piece in the developmental epigenetic puzzle. Sirtuins, and especially SirT1, are studied intensively for their roles in cell survival, apoptosis and differentiation, which has led to the development of many small molecule modulators to activate or inhibit their activity. The application SirT1 inhibitors centres mainly on cancer therapy, as many cancers overexpress SirT1, and SirT1 inhibitors induce apoptosis in cancer cell lines and *in vivo*. Sirtinol and some of its derivatives arrest cell cycling in breast and lung cancer (Mai et al., 2005). Both cambinol and tenovins are also able to inhibit cancer growth and to induce apoptosis (Heltweg et al., 2006; Lain et al., 2008). Salermide treatment leads to growth arrest and a pronounced apoptotic effect in various cancer cell lines without affecting a non-cancerous fibroblast line (Lara et al., 2009). Other potential applications for sirtuin inhibitors include Parkinson therapy (Outeiro et al., 2007) HIV replication blockade (Pagans et al., 2005) and *Leishmania infantum* treatment (Vergnes et al., 2005).

Our data suggest that SirT1 inhibition in differentiation enables the correct expression of specific developmental genes. This aspect could have an interesting application in the context of cancer therapy, as a specific goal of sirtuin inhibition could be to reactivate differentiation genes in cancers characterised by a low degree of differentiation. It could also have applications in regenerative medicine, to modulate and direct differentiation in stem cell-based therapies.

Given to the effects of SirT1 on longevity, rejuvenation and counteracting ageing, the pharmaceutical, "nutraceutical", and cosmetic industries are also interested in the development of small molecule SirT1 activators (Alcain and Villalba, 2009). Resveratrol, a natural compound present in traces in some red wines, has a demonstrated sirtuin-activating effect. This polyphenol mimics the anti-ageing effects of calorie restriction in simple organisms, and in mice fed a high-fat diet ameliorates insulin resistance, increases mitochondrial content, and prolongs survival (Baur et al., 2006b; Lagouge et al., 2006; Wood et al., 2004). Resveratrol might help in the treatment or prevention of obesity, as well as the ageing-related decline in heart function and neuron loss. As resveratrol has low bioavailability and specificity, medicinal chemists are developing new molecules to overcome these obstacles. SirT1 activators up to 1000 times more effective than resveratrol have recently been developed; they improve the response to insulin and increase the number and activity of mitochondria in obese mice. Human trials with an improved bioavailability formulation of resveratrol and with a synthetic SirT1 activator are in progress. The goal is to treat ageing-related diseases such as type 2 diabetes, and early data from mouse models are quite promising (Milne et al., 2007). Food supplements containing resveratrol at high concentrations are already available in pharmacies and nutrition shops. A commercial boom has centred the attention of the cosmetic

industry on SirT1 and its activators, and many laboratories are producing and selling preparations containing sirtuin activators (Moreau et al., 2007).

In essence, we basically propose that SirT1 has a positive role in stemness, aiding in the silencing of differentiation genes. This finding could be interesting, as one explanation of sirtuin activator action is that it contributes to maintaining a "stemness-like" status in cell populations involved in tissue regeneration. Our results could provide a useful starting point from which to study the potential teratogenicity of SirT1-modulating compounds, a factor to be considered for molecules rapidly coming into use in clinical practice and in everyday life.

In summary, this study addressed two epigenetic mechanisms responsible for downregulation of developmental gene expression in embryonic stem cells and modulation of correct spatio-temporal activation during cell differentiation (Fig. 42). The first is based on promoter DNA methylation, and is used by a subset of genes whose promoter has low CpG density and lacks bivalent mark/Polycomb-based signature in hESC; these genes are important for late lineage specification and are involved mainly in immune system functions. The second, SirT1-based mechanism, is more likely to be a modulatory effect that contributes with other repressive elements (e.g., Polycomb itself, as shown in mESC (Kuzmichev et al., 2005)) to the regulation of some developmental genes important in neuroectodermal and other lineage specification.

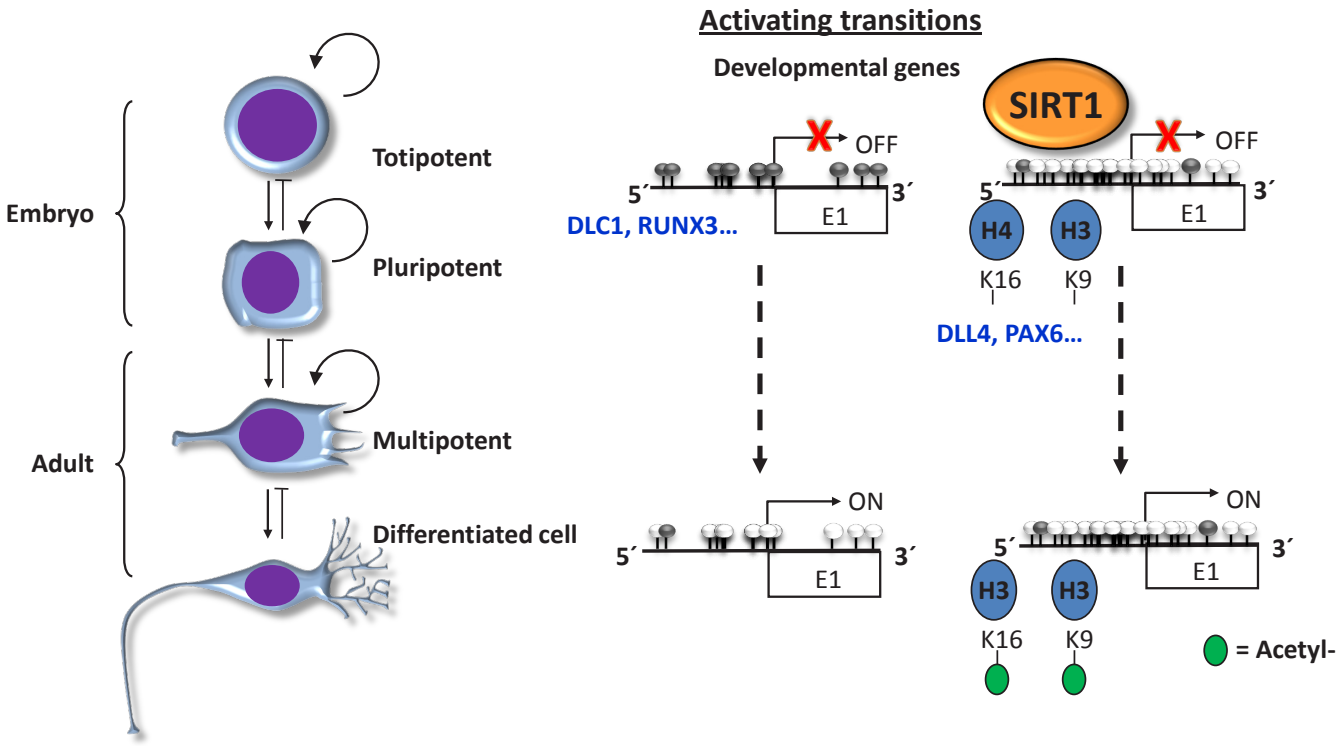


Figure 42. New mechanism in epigenetic regulation of hESC differentiation. This study highlights two undescribed activating mechanisms for developmental genes. Some "late differentiation" genes are hypermethylated in hESC and are activated through promoter demethylation during differentiation. Other genes are maintained silent in hESC, in part through SirT1-mediated promoter deacetylation, and are expressed later in development, modulated by SirT1 downregulation and promoter acetylation.

CONCLUSIONS

CONCLUSIONS

On the role of DNA methylation in ES cell differentiation

1. Promoter DNA methylation is an important gene regulation mechanism in embryonic and adult stem cells, as a means of developmental gene promoter repression.

2. The activation of a subset of differentiation genes, mainly immune system-related, during late ESC differentiation is mediated primarily by promoter DNA demethylation.

3. Twenty percent of the genes frequently hypermethylated in cancer (tumour suppressor genes, TSG) are also frequently hypermethylated in hESC. In contrast with other TSG frequently hypermethylated in cancer, these genes do not show bivalent domain histone marks (trimethyl-H3K4 and K27) at their promoters in hESC.

4. Promoter methylation can thus be regarded as alternative to bivalent domain-mediated repression for a subset of development-related/cancer hypermethylated genes.

On the role of histone modification in ES cell differentiation

5. The class III histone deacetylase SirT1 is regulated precisely during ES differentiation by a mechanism that involves the mRNA-binding protein HuR and the arginine methyltransferase CARM1. CARM1-dependent HuR methylation at Arg217 in pluripotent hESC increases HuR-dependent SirT1-mRNA stabilisation. CARM1 downregulation during ES differentiation results in a decrease in methyl-HuR and, consequently, in SirT1 donwregulation.

6. In pluripotent ESC, SirT1 binds to and epigenetically represses promoters of a subset of developmental genes, many of which are involved in neural fate specification. SirT1 downregulation during differentiation leads to activation of these genes through H4K16 and/or H3K9 acetylation, enabling the expression necessary for correct lineage specification. SirT1 might thus have an important role in establishment of the neuro-ectodermal layer.

7. SirT1 depletion in human and mouse ESC does not induce differentiation, but alters cell fate during *in vitro* difererentiation. SirT1 thus has a modulatory role in developmental gene regulation, contributing to correct spatiotemporal patterning in the differentiating embryo.

8. SirT1 overexpression in mouse ESC enhances the expression of pluripotency markers and delays their downregulation during differentiation, while also perturbing differentiation patterns.

RESUMEN ES ESPAÑOL

RESUMEN EN ESPAÑOL

INTRODUCCION

La epigenética fue definida por Conrad Waddington en 1942 como la ciencia que estudia cómo los genotipos dan lugar a fenotipos a través de cambios programados durante el desarrollo (Waddington, 1942). El desarrollo se considera el proceso que conduce a la formación de un organismo completo a partir de una sola célula mediante cambios progresivos. Después de la fecundación del óvulo, el cigoto se divide dando lugar a blastómeros, células consideradas totipotentes, ya que tienen el potencial de originar cualquier tipo celular diferenciado. En el estadío de dieciséis células, la mórula está constituida por células externas que dan lugar al trofoblasto, y por un pequeño grupo de células internas del que deriva el proprio embrión, denominado masa celular interna (ICM) (Barlow et al., 1972). El aislamiento de la ICM en esta etapa y su propagación *in vitro* en condiciones adecuadas, conduce a la derivación de líneas de células madre embrionarias (Moon et al., 2006). Estas células se definen como pluripotentes, ya que, aunque pueden formar cualquier linaje de células del embrión, ya han superado el primer paso de diferenciación. En 1998 se derivaron células madre embrionarias humanas (hESC) demostrando que estas células podían ser propagadas indefinidamente en cultivo y diferenciadas a varios linajes celulares (Gearhart, 1998; Thomson, 1998). Las células madre son una herramienta muy útil hoy en día para el estudio molecular de las primeras fases del desarrollo y, en un futuro, podrían ser utilizadas para producir nuevas células funcionales en situaciones patológicas en las que está comprometida la función celular (Deb and Sarda, 2008).

El conocimiento de nuevos mecanismos moleculares que conciernen a la epigenética ha llevado a añadir nuevos conceptos a la definición original. Consideramos un proceso epigenético aquel que afecte a la expresión de genes sin que para ello se vea alterada la secuencia de nucleótidos, de forma que puedan ser heredados a través de la división celular (Holliday, 1987). En el núcleo de células eucariotas, el ADN está organizado en nucleosomas (Oudet et al., 1975), una estructura modular formada por 147 pares de bases de ADN de doble cadena que rodean a un octámero de histonas (compuesto por dos dímeros de histonas H2A-H2B y un tetrámero de histonas H3-H4). La maquinaria epigenética se encarga de determinar la accesibilidad de la información contenida en el ADN utilizando un código formado por modificaciones covalentes en el ADN o en las histonas (Kouzarides, 2007). Las variaciones que la maquinaria epigenética introduce en la estructura cromosómica determinan diferencias en la compactación de la cromatina que se correlacionan estrechamente con estados de actividad o inactividad génica.

Las histonas poseen colas peptídicas que sobresalen de los nucleosomas y pueden ser modificadas en muchos residuos de aminoácidos. Hay por lo menos 30 sitios potenciales de modificación de histonas para cada nucleosoma y ocho tipos de modificaciones (metilación, acetilación, fosforilación, ubiquitinación, sumoilación, ADP-ribosilación, isomerización de prolina y biotinilación), algunas de las cuales pueden tener diferentes configuraciones (por ejemplo, la lisina pueden ser mono-, di- o trimetilada). La información contenida en las distintas combinaciones de las modificaciones de las histonas determina el estado funcional del ADN ; este lenguaje suele ser denominado código de histonas (Jenuwein and Allis, 2001).

La metilación del ADN se produce principalmente en citosinas que preceden a guaninas (CpGs). Estos dinucleótidos CpG se distribuyen asimétricamente en el genoma. Las regiones densas son las denominadas “islas CpG”, y se encuentran en la región promotora de aproximadamente la mitad de todos los genes. Estas islas CpG generalmente se encuentran no metiladas en las células normales (Illingworth and Bird, 2009). La falta de metilación permite la expresión de estos genes. Durante el desarrollo, ciertas islas CpG están sometidas a modificaciones en el patrón de metilación relacionadas con la diferenciación de los tejidos y el desarrollo. La diferenciación de las células madre embrionarias humanas (hESC) exige la represión de los factores de transcripción implicados en el mantenimiento de la pluripotencia, así como la activación de los genes de desarrollo. Ambos procesos son dirigidos por determinados mecanismos epigenéticos. Un ejemplo del primer tipo es la hipermetilación del promotor de los genes encargados del mantenimiento de la pluripotencia, como NANOG y OCT4 (Lagarkova et al., 2006) (Fig. I-8). Hasta ahora, hay pocos estudios sobre el proceso de desmetilación en la activación de genes de desarrollo durante la diferenciación de células madre. Sin embargo, se conoce que estos genes se encuentran, en su mayoría, en un estado reprimido durante las primeras etapas de desarrollo, debido a un patrón específico de modificaciones de las histonas, denominado “dominio bivalente”, que consiste en la coexistencia de metilación de la lisina 27 y de la lisina 4 de la histona H3 (Bernstein et al., 2006) (Fig. I-8). Este estado de represión de la cromatina está mediado por proteínas del grupo de Polycomb (Lee et al., 2006a; Sparmann and Van

Lohuizen, 2006). Se describió además que esta modificación predispone a una hipermetilación aberrante de promotores de genes supresores tumorales (TSG) en cáncer (Fig. 39) (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007).

Otro aspecto importante de la epigenética es la acetilación de histonas, una modificación asociada con activación de la transcripción génica (Shogren-Knaak et al., 2006). Las enzimas histonas desacetilasas se encargan de eliminar la acetilación de los nucleosomas, ejerciendo así una acción inhibidora de la trascripción. La Sirtuina 1 (SirT1) es una lisina desacetilasa dependiente del co-factor NAD^+ que participa en múltiples procesos celulares, incluyendo la remodelación de la cromatina, el silenciamiento de la transcripción, la mitosis, las respuestas al estrés, la reparación del ADN, la apoptosis, el ciclo celular, la estabilidad genómica, la regulación de insulina, y el control de la longevidad (Vaquero, 2009), así como un papel destacado en el desarrollo. En mamíferos, la función de SirT1 está mediada por su actividad desacetilasa, no sólo sobre las colas de las histonas (principalmente K16 y K9 de la histona H3 (Pruitt et al., 2006; Vaquero et al., 2007b), sino también por su actividad sobre factores de transcripción clave, como la proteína p53 y factores de transcripción FOXO (*forkhead box protein*), entre otros (Guarente and Picard, 2005). Estudios recientes realizados sobre modelos de ratones sugieren que SirT1 es importante en la diferenciación de células madre, así como en la diferenciación neuronal y glial de precursores neuronales (Prozorovski et al., 2008) y en la diferenciación de mioblastos esqueléticos en respuesta a la disponibilidad de glucosa (Fulco et al., 2003). Además, los ratones deficientes en SirT1 presentan graves defectos neurales, incluyendo neuro exencefalia y trastornos de la morfogénesis de la retina (Cheng et al., 2003; McBurney et al., 2003b). Sin embargo, la función de SirT1 en la diferenciación de hESC está todavía sin explorar.

OBJETIVOS

Nuestro estudio se ha centrado en dos aspectos de la epigenética en células madre embrionarias: la metilación del ADN y la modificación de las histonas por la histona desacetilasa SirT1.

Las células madre embrionarias tienen un patrón epigenético especifico. Bibikova *et al*. describieron 23 genes cuyo estado de metilación del promotor era exclusivo de hESC (Bibikova et al., 2006). La metilación del ADN también se ha asociado a la inactivación estable de genes de pluripotencia durante la diferenciación de hESC. Ciertos genes de diferenciación tardía que poseen un promotor con baja densidad de CpG y que no presentan marca bivalente, se encuentran con frecuencia hipermetilados en células madre (Fouse et al., 2008; Meissner et al., 2008). Sin embargo, la activación de genes durante el desarrollo mediante desmetilación del promotor aún no ha sido completamente investigada. Nos centraremos en este punto para definir los genes metilados específicamente en hESC en relación con los tejidos normales. Como la hipermetilación del promotor de TSG es una característica de muchos tipos de cáncer (Esteller, 2008), se analizaron además las similitudes entre la metilación de promotores de genes entre células madre y cáncer, lo que puede ayudar a comprender el proceso de metilación aberrante de TSG en cáncer.

Estudios recientes realizados en hESC confirman que la mayoría de los genes de desarrollo se reprimen por los complejos represivos Polycomb (PRC) y por una combinación de marcas de histonas llamada dominio bivalente, mediada principalmente por PRC2 y otros complejos con actividad histona metiltransferasa (Bernstein et al., 2006). Aunque se haya caracterizado ampliamente el papel de la metilación de las histonas en la regulación genética del desarrollo, la acetilación de las histonas en este contexto está menos estudiada. La histona desacetilasa SirT1 fue anteriormente implicada en procesos de diferenciación de los tejidos, como músculo, tejido adiposo y neuronas (Fulco et al., 2003; Picard et al., 2004; Prozorovski et al., 2008). SirT1 está altamente expresada en células madre y colocaliza en la cromatina con los componentes de PRC (Kuzmichev et al., 2005). Los ratones deficientes (KO) para SirT1 muestran importantes problemas en el desarrollo, aunque en muchos casos superan las primeras etapas embrionarias (Cheng et al., 2003; McBurney et al., 2003b). Por lo tanto, se ha estudiado el papel de SirT1 en la diferenciación de hESC, para determinar su implicación en la modulación de la expresión génica a través de la modificación de la cromatina.

Los objetivos concretos de esta tesis doctoral fueron los siguientes:

1. Caracterizar el papel de la metilación del ADN en la diferenciación de células madres embrionarias humanas, centrando especialmente nuestra atención en la regulación génica mediada por desmetilación de promotores.

2. Definir el papel en la regulación génica desempeñado por la modificación de histonas por parte de SirT1 a lo largo de la diferenciación de células madre.

RESULTADOS y DISCUSIÓN

En primer lugar hemos analizado los patrones de metilación del DNA en la diferenciación de las hESC. Primero estudiamos la metilación de dos líneas de células madre embrionarias y de las mismas líneas diferenciadas *in vitro* mediante la técnica de la amplificación de secuencias inter-metiladas (AIMS). Esta técnica utiliza enzimas de restricción sensibles a metilación para distinguir la metilación del ADN. Identificamos 23 secuencias de las que solo ocho están metiladas de forma preferente en la diferenciación celular (Figura 1 y Tabla 1).

Posteriormente utilizamos una técnica que permite el estudio simultáneo de muchos sitios CpG localizados específicamente en promotores génicos: el array de metilación GoldenGate, Illumina, que contiene información sobre la metilación de 807 genes, muchos de ellos relevantes en cáncer (Figura 2 y Tabla S2). Comparando los resultados obtenidos en 8 líneas de hESC, 21 muestras de tejido primario normal (NPT) y 21 muestras de líneas de cáncer (CCL) encontramos que cada uno de estos tipos celulares posee su propia firma epigenética. El 34.69% de los genes analizados en el estudio están hipermetilados en hESC (Tabla S2). Un 12,5% de los genes presentes en este array se encuentra hipermetilado en hESC y se desmetila en al menos un tejido normal, confirmando así que el proceso de activación de genes mediante desmetilación de promotor es detectable y no es secundario en la diferenciación *in vivo*. Muchos genes además se hipermetilan en cáncer, de los cuales aproximadamente el 20% también lo está en células madre y se encuentra desmetilado por lo menos, en una muestra de tejido normal, indicando que en algún momento de la diferenciación, este gen será activado. En base a los resultados obtenidos, realizamos una clasificación de los genes hipermetilados en cáncer, comúnmente considerados genes supresores de tumor (TSG). Consideramos como TGS de clase A-I los que solo están hipermetilados en cáncer; Clase A-II los que también se hipermetilan en muchos tejidos a lo largo de la diferenciación; Clase B-I los que están metilados en líneas hESC y se desmetilan a lo largo de la diferenciación en todos los tejidos, y clase B-II los que están metilados en líneas hESC y se desmetilan específicamente solo en algún tejido (Figura 3 y Tabla 2 y S2). Estos datos demuestran que existe una proporción sustancial de los genes hipermetilados en cáncer que también está metilado en las primeras fases del desarrollo. Comparando en el mismo array los resultados de metilación de una muestra de células madre adulta, los precursores hematopoyéticos $CD34^+$, con las hESC, observamos que ~92% de los genes hipermetilados en hESC lo seguía estando en esta muestra, indicando que probablemente la hipermetilación de estos genes es importante para todas las células con características de células madre, y abriendo la posibilidad a que la hipermetilación de algunos de estos genes en tumores pueda ser el resultado de un fallo del proceso de desmetilación a lo largo de la diferenciación, en lugar de la adquisición *de novo* de metilación aberrante durante la trasformación tumoral (Figura 12, tablas S7). El hecho de que muchos genes pierdan metilación a lo largo del desarrollo también es un resultado interesante. Todos los genes de la clase B están hipermetilados y, por lo tanto, inactivos en hESC. Los de la clase B-I probablemente se desmetilan en fases muy tempranas del desarrollo, ya que se encuentran desmetilados en la mayoría de los tejidos adultos. Los de la Clase B-II se desmetilan específicamente en uno o pocos tejidos, predisponiéndose así para una expresión especifica de tejido. Estos genes están involucrados en procesos de diferenciación tardía (Tabla S16). Comparando nuestros resultados con los datos publicados de otros arrays observamos que mientras que los genes de clase A se comportan como está previsto para los TGS, estando en su mayoría pre-marcados en hESC con proteínas del grupo Polycomb y dominio bivalente, los de clase B, y particularmente los de clase B-II no suelen tener ninguna de estas marcas. Esto significa que la hipermetilación de estos genes puede ser un proceso alternativo para la represión al que fue previamente propuesto, el dominio bivalente (Figura 3 A y B, Tablas S4 y S5). También pudimos reproducir la desmetilación de promotores en un modelo *in vitro* de diferenciación de hESC en el que diferenciamos la línea Shef-1 a neuronas y a células mesodérmicas (fibroblastos; Figura 9 y Tabla S6). Encontramos, respetivamente, 12 y 25 genes que pierden metilación durante estas diferenciaciones, y sólo tres de ellos eran comunes a los dos linajes, indicando que la desmetilación de genes puede ser un proceso específico de linaje durante la diferenciación. Esta misma observación se pudo hacer en estadios más avanzados de diferenciación, comparando los genes hipermetilados en precursores hematopoyéticos que se desmetilan específicamente en linaje linfoide (9) y mieloide (16) (Figura 12 y Tabla S8). El patrón de metilación de muchos de estos genes analizado en el array fue validado por secuenciación de bisulfito (Figuras 4, 5, 6, 7, 8, 10 y 12). Además se cuantificó la expresión del ARN mensajero de estos genes por la reacción en cadena de la polimerasa (PCR) cuantitativa, relacionando metilación génica con represión transcripcional. Sin embargo, la falta de metilación de un gen no siempre implicaba una activación transcripcional (Figuras 4, 5, 6, 7, 8, 10 y 12). Esto confirma que en muchos casos es necesaria la implicación de otros factores epigenéticos para la inducción de la transcripción.

Realizamos un estudio aún más amplio y detallado de la

metilación génica en hESC, empleando otro array de metilación que contiene información sobre más de 14000 genes, casi la totalidad de los genes regulados por metilación en el genoma humano (Tabla S9). Comparamos 10 muestras de líneas de hESC y 10 muestras de tejidos primarios diferenciados y observamos que un 35% de los genes de todo el array se encuentra hipermetilado en hESC y que un 32% de estos últimos pierde metilación en al menos un tejido (Tabla S10). Mediante la interfaz de la red DAVID, una herramienta de estudio de la ontología génica, buscamos una función común para estos genes. Un porcentaje significativamente alto de estos genes ejerce funciones asociadas al sistema inmune y respuesta inflamatoria (Tabla 3). Además, muchos de ellos codifican para proteínas de membrana o secretadas. Esta observación encaja con la anterior, que los genes hipermetilados en hESC suelen tener funciones específicas en el desarrollo tardío. En el análisis de los datos ofrecidos por este array en muestras diferenciadas *in vitro* desde las hESC a células mesodérmicas, obtuvimos resultados muy parecidos: más de 200 genes, de los que muchos están relacionados con la respuesta inmune, se desmetilan específicamente durante este tipo de diferenciación (Tablas 4 y S11). Este resultado indica que estos genes parecen desbloquear su expresión en estadios iniciales de la diferenciación mesodérmica y que después, en el linaje hematopoyético, se activan probablemente a través de otros mecanismos epigenéticos. Por último se compararon los perfiles de metilación de promotores de líneas hESC cultivadas a corto o largo pase de cultivo, siendo la diferencia de número de pases de alrededor de 100. En estas líneas se observó que alrededor de un 2 % de los genes del array estaban sujetos a hipermetilación, y muy pocos a desmetilación. Se detectó una familia particular de genes, los factores de transcripción con un dominio de dedo de zinc, que parecen estar sujetos a hipermetilación en el proceso de adaptación al cultivo de las células madre (Tablas 5 y S12).

En segundo lugar, estudiamos la regulación de la estructura cromatínica por acetilación de histonas en la diferenciación de hESC. Datos de espectrometría de masas "top-down" nos mostraron que los niveles globales de la forma mono y di-acetilada de la histona H4 aumentaban sensiblemente en las primeras fases de la diferenciación *in vitro* de hESC (Figura 14). Ya que la histona desacetilasa SirT1 es la única que se ha demostrado que desacetila la forma monoacetilada de la histona H4 (en la lisina 16) (Vaquero et al., 2007b), decidimos estudiar la expresión de la enzima SirT1 en hESC. Los niveles de expresión de SirT1 son elevados en las hESC y dicha expresión, tanto a nivel de ARN como de proteína, decae rápidamente en la diferenciación *in vitro* a cuerpos embrioides (EB) y a fibroblastos (F-L) (Figura 15 y 17). SirT1 parece estar localizada principalmente en el núcleo, excepto en las células en fase de mitosis, en las que se encuentra difusa por toda la célula (Figura 16). En primer lugar, nos planteamos explicar las razones por la que la expresión de esta enzima descendía durante la diferenciación. No encontramos diferencias a nivel de su promotor tanto en metilación (Figura 18) como en actividad transcripcional, analizada mediante la acetilación global de las histonas H3 y H4 (Figura 19). Sin embargo, como los niveles de ARN mensajero descendían, decidimos evaluar la estabilidad de su tránscrito. Detectamos que el tránscrito de SirT1 se hace más inestable tan solo a los tres días de diferenciación (Figura 20). Había sido descrito que la proteína HuR regula la estabilidad del tránscrito de SirT1 en cáncer (Abdelmohsen et al., 2007). Analizamos, por lo tanto, la posible implicación de esta proteína que estabiliza ARN en células madre. Observamos que la unión de HuR al transcrito de SirT1 sufre una repentina bajada en la diferenciación (Figura 21A) y que la eliminación de HuR (generada mediante interferencia del ARN) determina una bajada de la expresión de SirT1 (Figura 21B). Como la actividad de HuR está regulada en otros sistemas por medio de su localización sub-celular y su fosforilación, analizamos estos procesos en diferenciación, sin encontrar ninguna diferencia significativa (Figuras 23, 24 y 25A). Como se había descrito previamente que la metilación de HuR en la arginina 217 determinaba un incremento de la unión de HuR a sus dianas en un sistema de neutrófilos de ratón (Li et al., 2002a), analizamos los niveles de metilación de este residuo en nuestro sistema y pudimos detectar una bajada de metilación de HuR durante la diferenciación (Figura 25B). Este residuo está metilado por la arginina metiltransferasa CARM1 (Li et al., 2002a), así que estudiamos su implicación en los fenómenos observados. Este enzima se regula negativamente durante la diferenciación y su eliminación mediante interferencia de ARN provoca una bajada de los niveles de HuR metilado y consecuentemente de SirT1 (Figura 26). Comprobamos además que la metilación de la arginina 217 de HuR era realmente responsable de un cambio de su actividad en la unión al transcrito de SirT1 en nuestro sistema. Para aquello transfectamos las células con unos plásmidos que codifican para mutantes de la proteína HuR en el residuo 217, convertido en lisina y en alanina. En ambos casos observamos que las proteínas mutantes expresadas en hESC unían el ARN mensajero de SirT1 con una eficiencia mucho menor que la proteína salvaje (Figura 27).

A continuación estudiamos las dianas moleculares de SirT1 en hESC. Para ello realizamos un estudio genómico de los sitios de unión de SirT1 a secuencias de ADN mediante un

experimento de inmuno-precipitación de cromatina (ChIP), usando un anticuerpo especifico de SirT1 e hibridando el DNA inmuno-precipitado en un array que contiene los promotores de prácticamente todos los genes humanos descritos. Encontramos así 353 genes a los que la SirT1 se une en hESC (Tabla S13). Analizando mediante algoritmos de ontología génica la función de estos genes encontramos que estaban relacionados con funciones de desarrollo y diferenciación celular (Tabla 6). Por lo tanto, formulamos la hipótesis de que SirT1 contribuye a mantener silenciados genes de diferenciación en células madre. Para comprobar esta hipótesis elegimos 10 genes de desarrollo, y dos controles negativos de genes expresados de forma constitutiva y medimos en ellos, a través de ChIP cuantitativo, la unión de SirT1 y los niveles de acetilación de los residuos lisina 16 de la histona H4 y lisina 9 de la histona H3, las dos dianas mejor caracterizadas de esta histona desacetilasa (Figura 30 y 31). Detectamos en los promotores de estos genes un enriquecimiento de SirT1 y un aumento de los niveles de acetilación de las histonas, paralelo a un aumento de expresión en la diferenciación (Figura 32). Además, silenciando la expresión de SirT1 mediante interferencia de ARN detectamos en muchos casos un aumento de la expresión de estos genes en hESC (Figura 30).

Finalmente nos propusimos investigar la posible función de SirT1 en la diferenciación celular. Para ello observamos el comportamiento de las células madre embrionarias después de la interferencia de SirT1 (Figura 33). Las células madre no presentaron ningún cambio fenotípico aparente provocado por la ausencia de SirT1. Además, los marcadores de pluripotencia y de diferenciación no sufrieron una modificación relévate en estas condiciones (Figura 34). Sin embargo, cuando indujimos la diferenciación de estas células a EB pudimos observar una alteración más importante de los niveles de marcadores de diferenciación (Figura 34). Analizamos además el comportamiento de células madre de ratón en las que la expresión de SirT1 está silenciada por eliminación del gen (Sirt1 KO) o aumentada por inserción de copias adicionales del gen (SuperSirt1; Figura 35). El hecho de que estas células puedan generar ratones vivos, que en el caso de los SirT1 KO presentan problemas de desarrollo pero en muchos casos llegan al nacimiento (Cheng et al., 2003), y en el caso de SuperSirt1 no presentan ningún fenotipo especifico de desarrollo, nos indicaba que SirT1 no es esencial para los procesos clave del desarrollo (Pfluger et al., 2008). Probablemente Sirt1 desempeña, más bien, un papel en la modulación de la expresión de los genes de desarrollo. Observamos que la ausencia de Sirt1 tiene un efecto moderado sobre las células en su estadio indiferenciado, pero un efecto más acusado cuando se induce su diferenciación. Sin embargo, las células madre SirT1 KO mostraban una reducción moderada en la expresión de los seis marcadores de pluripotencia analizados (FGF4, Nanog, Nodal, Rest, Tdfcp2l1 y Tdgf1), mientras que las células madre SuperSirt1 tienden a sobre-expresar estos marcadores de pluripotencia (Figura 38). Además, aunque las células Sirt1 KO diferenciadas a EB no mostraron cambios claros en la expresión de marcadores de pluripotencia, los EB de la línea Super-Sirt1 mantuvieron una expresión notable de estos marcadores que, en la mayoría de los casos, era comparable a su expresión en las células madres salvajes. Estos resultados sugieren que la función de Sirt1 en células madre podría no ser idéntica en ratón y humano y que, a diferencia de las células madre humanas, Sirt1 puede contribuir al mantenimiento de la pluripotencia de células madre de ratón. Durante la diferenciación, se observó que tanto la ausencia como la sobreexpresión de Sirt1 dan lugar a una profunda alteración de la mayoría de los marcadores de desarrollo analizados (Fig. 37); observación que se relaciona con nuestros resultados en las células madre humanas. Los marcadores que mostraron alteraciones importantes son los correspondientes a la capa neuro-ectodérmica (Neurod1, Syp y Nes), que se sobreexpresan en Sirt1 KO EB y se regulan negativamente en Super-Sirt1 EB. Estos resultados indican que SirT1 podría tener un papel importante en la creación de la capa neuro-ectodérmica.

CONCLUSIONES

Respecto al papel de la metilación del ADN en la diferenciación de células madre

1. La metilación del ADN en los promotores es un mecanismo importante de regulación génica en células madre adultas y en el desarrollo embrionario como herramienta de represión de los genes de desarrollo.

2. La activación durante la diferenciación tardía de células madre de determinados genes de desarrollo, principalmente los relacionados con el sistema inmune, está mediada fundamentalmente por la desmetilación de sus promotores.

3. El 20% de los genes hipermetilados frecuentemente en cáncer (genes supresores tumorales, TSG) están también frecuentemente hipermetilados en células madre embrionarias. A diferencia de otros TSG, en las células madre estos genes no presentan en sus promotores la marca de histonas de dominio bivalente (K4 y K27 de la histona H3 trimetiladas) característica.

4. La metilación de los promotores se puede considerar una alternativa a la represión génica mediada por el dominio bivalente para un subconjunto de genes de desarrollo frecuentemente hipermetilados en cáncer.

Respecto al papel de la modificación de las histonas en la diferenciación de células madre

5. La histona desacetilasa de clase III SirT1 está regulada durante la diferenciación de células madre embrionarias por un mecanismo que involucra a la proteína HuR, que se une al ARNm de SirT1 , y la arginina metiltransferasa CARM1. La metilación de HuR mediada por CARM1 en la Arg217 aumenta la estabilidad del ARNm de SirT1 en células madres pluripotentes. El nivel de expresión de CARM1 desciende durante la diferenciación de células madre, provocando así una disminución de HuR metilado y, consecuentemente, una disminución de SirT1.

6. En células madre embrionarias, SirT1 se une a los promotores de un subconjunto de genes del desarrollo, muchos de ellos implicados en la diferenciación neural, y los reprime epigenéticamente. La disminución de los niveles de Sirt1 durante la diferenciación provoca la activación de estos genes mediante la acetilación de la Lys16 de la histona H4 y/o de la Lys9 de la histona H3, permitiendo la expresión génica necesaria para la diferenciación celular al linaje correcto. Por lo tanto, SirT1 podría desempeñar un papel importante en la formación de la capa neuro-ectodérmica.

7. La eliminación de SirT1 en células madre humanas y murinas no induce diferenciación, sin embargo, altera el destino celular durante la diferenciación in vitro. Por lo tanto, SirT1 posee una función moduladora en la regulación de los genes de desarrollo, contribuyendo al correcto patrón espacio-tiempo de expresión génica durante el desarrollo embrionario.

8. La sobreexpresión de Sirt1 en células madre murinas aumenta la expresión de los genes implicados en la pluripotencia y retrasa su disminución durante la diferenciación, alterando así los patrones de diferenciación.

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INVITED BOOK CHAPTERS

Epigenetic Drift and Aging

Ester Lara, **Vincenzo Calvanese**, and Mario F. Fraga

In book: Epigenetics of Aging, Tollefsbol, Trygve O. (Ed.) 2010, XII, 448 p. 55 illus., 20 in color., Hardcover ISBN: 978-1-4419-0638-0

Epigenetic code and self-identity

Vincenzo Calvanese, Ester Lara and Mario F. Fraga

In book: Ancient Origin Of Self-Sensing In Nature, at Landes Biosciences (Springer-Verlag distribution). *in press*